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Epigenetic Effects of Iron and Zinc Deficiency in *Saccharomyces cerevisiae*

Investigation of heat and oxidative stress resistance for discovering phenotypic alterations induced by iron and zinc deficiency

Master's thesis in the master degree program in biotechnology

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THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

Individual variations in health and disease cannot only be explained by genetics, but rather by a combination of genetics and epigenetics. Epigenetics is defined as the study of heritable changes in gene expression that occur without any changes in the DNA sequence. Epigenetic mechanisms contribute to reversible changes in gene expression which in turn affect the phenotype of the organism. The epigenome is inherited to the daughter cells during mitosis but epigenetic patterns can also be inherited transgenerationally. Most environmental factors like nutrition, stress and endocrine disruptors, do not cause genetic mutations but they can promote epigenetic alterations. Nutrition is one of the main environmental factors that can induce epigenetic changes in an organism and thereby influence gene expression and health. The aim of this project was to study the impact of iron and zinc deficiency on the epigenome and phenotype of *S. cerevisiae* by investigating the resistance against heat and oxidative stress. Iron deficiency induced a change in the phenotype of *S. cerevisiae*, which was observed as reduced oxidative stress resistance and inherited for three generations. Zinc deficiency also resulted in a phenotype with reduced oxidative stress tolerance that was inherited for two generations. In addition, a phenotype with reduced heat stress resistance was induced by zinc deficiency, but this alteration was not shown to be reversible. The observed phenotypic changes were most likely caused by epigenetic modifications, but this have to be confirmed in further studies by examining the epigenome on a molecular level. Because of these changes in phenotype, it was also concluded that *S. cerevisiae* can be used as an efficient model organism for studies in nutritional epigenetics, with the goal to improve health and prevent diseases.

Keywords: Epigenetics, Phenotype, *Saccharomyces cerevisiae*, Iron deficiency, Zinc deficiency, Heat stress, Oxidative stress, Hydrogen peroxide

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TABLE OF CONTENTS

1 INTRODUCTION

Research has shown during the recent decades that individual variations in health and disease cannot only be explained by genetics, but rather by a combination of genetics and epigenetics (Dolinoy *et al*., 2007). Epigenetics is defined as the study of heritable changes in gene expression that occur without any changes in the DNA sequence (Wolffe & Matzke, 1999). Epigenetic mechanisms include DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs. These mechanisms contribute to changes in gene expression which in turn affects the phenotype (Jiménez-Chillarón *et al*., 2012). Epigenetic processes can also change the expression of genes associated with conditions and diseases such as Alzheimer's, cancer and cardiovascular diseases (Lista *et al*., 2013, Soubry, 2015), which makes epigenetics a highly essential field of research.

The environment has an important role in regulating phenotype formation and disease etiology through epigenetic processes. There are many biological observations where a significant impact of the environment on disease etiology cannot be explained simply by classical genetic mechanisms (Guerrero-Bosagna & Skinner, 2012). Most environmental factors like nutrition and endocrine disruptors, do not cause genetic mutations but they can promote epigenetic alterations. Epigenetic patterns are inherited to daughter cells during mitosis but can also be inherited transgenerationally. Environmental factors can give rise to changes in the germline epigenome, which allows for the acquired phenotype to be inherited to the offspring and subsequent generations (Skinner *et al*., 2010).

Nutrition is one of the main environmental factors that can induce epigenetic and phenotypic changes, both through direct exposure and through transgenerational inheritance of epigenetic traits (Niculescu, 2012). Nutrients and bioactive food components can stabilize the genome through epigenetic mechanisms and they may also reverse maladaptive epigenetic patterns, yielding positive health effects. In contrast, malnutrition and nutrient deficiencies can generate epigenetic modifications that lead to silencing or activation of disease-related genes (Mazzio & Soliman, 2014). Epigenetic changes are though potentially reversible, which creates possibilities for dietary interventions that could be used to improve health and prevent diseases.

Billions of people all over the world are suffering from micronutrient deficiencies such as iron, iodine, vitamin A and zinc deficiency. Micronutrients are needed by the body in very small quantities but they are nonetheless essential and must be supplied in the diet. Deficiency disorders can be caused by insufficient intake or low absorption and bioavailability of certain nutrients. Micronutrient deficiencies affect the health of individuals, but also the development potential of societies and national economies (World Health Organization, 2002). Iron deficiency is the most prevalent and widespread nutritional disorder in the world. It affects around 2 billion people and it is common in both developing and industrialized countries (World Health Organization, 2016). Zinc deficiency is not as widespread as iron deficiency and affects mostly people in developing countries. It is estimated to affect one-third of the world's population but ranges from 4 % to 73 % across different regions. Mild to moderate zinc deficiency is rather common in the world even though severe zinc deficiency is rare (World Health Organization, 2002).

Several studies have shown in the recent years how dietary factors can modulate the establishment and maintenance of epigenetic patterns and thereby influence gene expression and health (Jiménez-Chillarón *et al*., 2012; Soubry, 2015). The knowledge about nutritional epigenetics is though inadequate and more studies are required in order to gain better understanding of the impact of

nutrients and bioactive food components on the epigenome and health of current and future generations. Only a few studies have investigated the relation between iron and zinc deficiency, disease and epigenetic processes, and because of the high prevalence of these micronutrient deficiencies in the world, there is a great need for more research in this field.

1.1 Aim and research questions

The aim of the project was to study the impact of iron and zinc deficiency on the epigenome and phenotype of *S. cerevisiae* by investigating the resistance against heat and oxidative stress. The project was intended to be a preliminary study for further research in nutritional epigenetics.

The goal of the project was also to answer the following research questions:

- Which iron and zinc concentrations cause iron and zinc deficiency and heavily reduced growth rates in *S. cerevisiae*?
- What is the optimal temperature for exposing *S. cerevisiae* to heat stress and studying the survival?
- What is the optimal concentration of hydrogen peroxide for exposing *S. cerevisiae* to oxidative stress and studying the survival?
- How does the resistance against heat stress and oxidative stress differ between *S. cerevisiae* cultured in iron and zinc limited medium and *S. cerevisiae* grown in complete medium?
- Can iron and zinc deficiency induce epigenetic alterations and thereby changes in phenotype?
- Will changes in phenotype be visible through differences in stress resistance, if the growth medium is restored to non-limited iron and zinc concentrations after several generations with iron and zinc deficiency?
- Are the changes in phenotype caused by epigenetic alterations that are inheritable and reversible?
- Is *S. cerevisiae* an efficient model organism to use for studies in nutritional epigenetics?

1.2 Limitations

In this study, *S. cerevisiae* was cultured in batches, which means that the iron and zinc concentrations were not kept at a constant level. But it was still possible to develop iron and zinc deficiency in the cultures and subsequently investigate the heat and oxidative stress resistance of the cells. It might have been more optimal to culture the cells in a chemostat, but this was not chosen as a method due to limited time and equipment. The epigenetic mechanism of DNA methylation is not present in *S. cerevisiae* (Morselli *et al*., 2015) and therefore it was not possible to evaluate the effects of iron and zinc deficiency on DNA methylation in this study. Due to the limited amount of time, it was not possible to confirm any specific epigenetic changes on a molecular level by analyzing gene expression and histone modifications.

2 THEORETICAL BACKGROUND

The following chapter includes a more thorough background to the field of epigenetics, with focus on nutritional epigenetics and iron and zinc deficiency. The chapter also contains a theoretical description about the methods used in this study, including batch cultivation, optical density, heat stress and oxidative stress.

2.1 Iron – biological function and deficiency

Iron is an important element that is necessary for life due to its redox reactivity. The two stable and interchangeable oxidation states of iron in biological systems are primarily ferrous (Fe^{2+}) and ferric (Fe^{3+}). About 60 % of the iron in the human body is utilized in the protein hemoglobin in the red blood cells that transport oxygen, which is critical for cell respiration. 5 % of the iron is found in myoglobin, which is a protein that is required for oxygen storage in muscles. Another 5 % is used in various heme and nonheme enzymes, whereof some are essential constituents of the electron transport chain, which is important for energy production. Ferritin and hemosiderin store the remaining 30 % of the iron in the body. Only less than 0.1 % of the iron is bound to the iron transport protein transferrin (Gibney & Nutrition Society, 2009).

Too low intake of iron can cause iron deficiency anemia, which is a condition with insufficient amounts of hemoglobin in the red blood cells and less oxygen will therefore be transported throughout the body (Scanlon & Sanders, 2007). Symptoms of iron deficiency anemia are paleness, fatigue, dyspnea and impaired immune defense. The recommended daily intake of iron is 9 mg for adults. Children and pregnant women have higher demands of iron, up to 15 mg per day, and are therefore more severely and commonly affected by iron deficiency. Good dietary sources of iron are liver, meat, fish, eggs, wholegrain products and spinach (Livsmedelsverket, 2016a). It is also important to make a distinction between intake and absorption since inhibitors like phytates and polyphenols can reduce the absorption of iron even if the intake is sufficient (Kim *et al*., 2011).

2.2 Zinc – biological function and deficiency

Zinc is present in its divalent state (Zn^{2+}) in biological systems and is not a redox-active metal unlike iron. It has several functions including catalytic, structural and regulatory functions. The folding and activity of a large number of proteins are dependent on zinc, and hundreds of metalloenzymes use zinc as a cofactor. Important structural functions of zinc are both in metalloenzymes and zinc finger motifs in proteins, which can bind to DNA and regulate gene expression. In addition, zinc is required by protein kinases involved in signal transduction processes (Gibney & Nutrition Society, 2009) and it is also an important part of the immune system (Shankar & Prasad, 1998). A study made by Andreini *et al*. (2006) showed that about 10 % of the human proteins contain zinc, which further underlines the importance of zinc. High concentrations of zinc are though toxic to cells and intracellular concentrations of zinc are therefore tightly regulated (Okumura *et al*., 2011).

About 60 % of the zinc in the human body is present in skeletal muscle, 29 % in bone, 6 % in skin and 5 % in liver. Zinc deficiency can affect human health in numerous ways. Symptoms of severe zinc deficiency are growth retardation, skeletal and sexual immaturity, neuropsychiatric disturbances, diarrhea, dermatitis, alopecia, impaired immune defense and loss of appetite (Gibney & Nutrition Society, 2009). The recommended daily intake of zinc is 7 mg for women and 9 mg for men, but children and pregnant women need up to 12 mg per day. Dietary sources that contain zinc are liver, meat, dairy foods, wholegrain products, eggs and nuts (Livsmedelsverket, 2016b). As for iron, it is important to

consider that inhibitors like phytates and fibers in the diet could affect the absorption of zinc (Foster *et al*., 2012; Gharib *et al*., 2006).

2.3 Epigenetics

A multicellular organism contains cells that are genetically homogenous, but structurally and functionally heterogeneous due to qualitative and quantitative differences in gene expression. Control of gene expression is hence essential for differentiation and development. Many of the differences in gene expression which characterize differentiated cells are established during development and are subsequently retained when the cells divide during mitosis. Thereby, cells inherit both genetic information and information which is not encoded in the DNA sequence, called epigenetic information. Simply described, this epigenetic information decides whether the expression of a gene is turned on or off (Jaenisch & Bird, 2003).

2.3.1 The structure of chromatin

In eukaryotic chromosomes, proteins and DNA together form a complex called chromatin. The proteins are divided into two classes: histones and non-histone chromosomal proteins. Histones are highly conserved eukaryotic proteins which are responsible for the first level of chromosome packaging. Histone proteins are present in DNA-protein complexes called nucleosomes. Each nucleosome core particle consist of a histone octamer and double stranded DNA, which is 147 nucleotide pairs long and wrapped around the histone octamer. The histone octamer consist of two of each of the histones H2A, H2B, H3 and H4. Between each nucleosome core particle, there is a region with linker DNA that can be up to 80 nucleotides long. The structure of the nucleosomes shortens the chromatin into one-third of its initial length. Another histone protein, called H1, is also crucial for compacting the chromatin by binding to the nucleosomes and changing the path of the DNA that exits from the nucleosomes (Alberts, 2008). The condensed packaging of DNA preserves the genetic information, but the chromatin structure must also be dynamic and receptive to remodeling in order to be able to assist and regulate events such as replication, transcription and DNA repair in various cell types. There are several different epigenetic mechanisms that can change and modify the chromatin structure and thereby affect the accessibility and reading of the underlying DNA sequence. The epigenetic mechanisms include DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs and the occurrence of these chemical modifications constitute the epigenome of an organism (Jiménez-Chillarón *et al*., 2012; Murr, 2010).

2.3.2 DNA methylation

Cells have the capacity to methylate and demethylate their DNA, which in turn influences the gene expression. Methylations are catalyzed by a family of enzymes named DNA methyltransferases (DNMTs) (Gibney & Nolan, 2010). The mechanisms for demethylation are though less understood but are thought to involve the ten-eleven translocation (TET) enzyme family and thymine DNA glycosylase (TDG) (Kohli & Zhang, 2013). Methylation of the C-5 position of cytosine is a covalent, reversible modification of DNA and that is one of the most studied epigenetic mechanisms. In mammals, cytosine methylation occurs mainly at CpG dinucleotides, which are cytosines linked to the 5' end of guanines through a phosphodiester bond. The methylation allows normal hydrogen bonding between the bases but changes the biochemical properties of DNA, since the methyl groups protrude into the major groove of DNA, which is the space where the DNA backbones are furthest apart and the DNA is more accessible for protein interactions (Gibney & Nolan, 2010; Prokhortchouk & Defossez, 2008). DNA methylation is generally associated with gene repression, which can occur through three different

mechanisms. The added methyl groups can either inhibit the recognition of specific DNA sequences by transcription factors, or enable binding of proteins with repressive potential that recognize methylated cytosines (Klose & Bird, 2006). The methyl groups could also alter the chromatin structure and the accessibility of DNA for DNA binding proteins (Zhang & Pradhan, 2014). CpG dinucleotides are less common than expected in most parts of mammalian genomes but they occur with a high frequency in some regions called CpG islands, which are regions longer than 200 base pairs containing a higher than expected abundance of CpG sites. The CpG islands are unevenly distributed through the genome and many are concentrated to promoter regions, which mostly remain unmethylated to maintain transcription of active genes. CpG islands are also frequently occurring within genes that generally are involved in DNA repair and cell cycle regulation (Gibney & Nolan, 2010; Zhang & Pradhan, 2014).

2.3.3 Histone modifications

Each histone in a nucleosome has an N-terminal amino acid tail that extends out of the nucleosome. These tails are subject to a variety of post-translational modifications (PTMs) that can alter the chromatin structure and function. 21 different types of histone PTMs have so far been identified on several different amino acids: mono-, di- and trimethylation, acetylation, phosphorylation, ubiquitination, SUMOylation, biotinylation, propionylation, butyrylation, formylation, hydroxylation, malonylation, succinylation, crotonylation, hydroxylisobutyrylation, glutarylation, O-GlcNacylation, citrullination, ADP-ribosylation and proline isomerization (Huang *et al*., 2015; Jiménez-Chillarón *et al*., 2012). Three mechanisms, similar to those related to DNA methylation, are thought to regulate the structure and function of chromatin through histone PTMs. Histone PTMs can directly affect the packaging of chromatin by altering the charge of histones or the interactions between the nucleosomes, and thereby changing chromatin structure and accessibility of regulatory DNA-binding proteins (Zhang & Pradhan, 2014). Histone PTMs can also attract and recruit certain effector proteins that recognize specific histone marks, or they can inhibit bindings between proteins and chromatin (Gibney & Nolan, 2010).

Since there is a large number of different histone modification types and sites, the number of potential combinations of PTMs and their biological outcomes is huge (Nikolov & Fischle, 2013). Many of the PTMs are connected to transcriptional activation or repression depending on the type and location of the modification. As examples, acetylation of lysine residues usually correlates with transcriptional activation and lysine deacetylation with transcriptional repression. Lysine methylation can be involved in both transcriptional activation and repression depending on the location of the lysine within the histone tail. In addition, some PTMs are also involved in DNA repair and replication (Huang *et al*., 2015). Histone PTMs are reversible with diverse families of enzymes that regulate the addition and removal of the different modifications, and new enzymes are still being discovered (Berger, 2007; Huang *et al*., 2015).

2.3.4 Chromatin remodeling

In addition to DNA methylation and histone modifications, the structure of chromatin can also be altered by ATP-dependent chromatin remodeling complexes, which are enzymes that utilize the energy from ATP hydrolysis to slide nucleosomes along DNA, alter the conformation of nucleosomal DNA and remove or change the composition of nucleosomes (Narlikar *et al*., 2013). These changes make chromatin more available for proteins that require direct access to DNA or histones during different cellular processes such as transcription, recombination, replication and repair (Hargreaves & Crabtree, 2011). The chromatin remodeling complexes can change the composition of histone

octamers by incorporating variants of the core histones, which could alter the stability of the nucleosome or introduce new possibilities for posttranslational modifications. Several of the complexes also contain additional enzymatic subunits that can regulate histone modifications directly. The ATP-dependent chromatin remodeling complexes are divided into four subfamilies: SWI/SNF, ISWI, INO80 and CHD. Each family possesses a characteristic ATPase subunit and also unique motifs that can bind to specific nucleosomal subunits (Swygert & Peterson, 2014). Mutation or deletion of chromatin remodeling complexes in adults often results in apoptosis or tumorigenesis due to impaired cell cycle control (Hargreaves & Crabtree, 2011).

2.3.5 Non-coding RNAs

Non-coding RNA is the part of the RNA that is transcribed from DNA but not translated into proteins (Maulik & Karagiannis, 2014). The size of the non-coding RNAs ranges from very short to extremely large transcripts. Non-coding RNA molecules can be classified according to their length, cellular location and function (Gibney & Nolan, 2010). The unique sequences and the complementarity with both DNA and mRNA make the non-coding RNAs into excellent candidates for delivering epigenetic enzymes to specific loci (Szyf, 2015). There are several different classes including long non-coding RNAs, microRNAs, small interfering RNAs and PIWI-interacting RNAs. Many of the long non-coding RNAs can recruit chromatin remodeling complexes and thereby facilitate chromatin modifications and changes in gene expression. The long non-coding RNAs play essential roles in several processes such as cell differentiation, X chromosome inactivation, disease progression processes and organ development. MicroRNAs and small interfering RNAs are both involved in posttranscriptional gene silencing through degradation of their target mRNAs (Zhang & Pradhan, 2014). MicroRNAs are important for cell differentiation and dysfunctional regulation of these RNAs is associated with cancer and neurological diseases such as Alzheimer's and Parkinson's (Junn & Mouradian, 2012; Maulik & Karagiannis, 2014). PIWI-interacting RNAs are associated with the PIWI proteins and are involved in histone methylation and gene silencing (Ross *et al*., 2014). Both small interfering RNAs and PIWI-interacting RNAs can target specific loci for DNA or histone methylation (Szyf, 2015).

2.3.6 Interplay between different epigenetic mechanisms

The epigenetic mechanisms can independently regulate cellular processes, but numerous studies have also shown that there exist several interactions between the different mechanisms (Murr, 2010). Interactions between DNA methylation and histone modifications could influence the gene expression and result in a common outcome. For example, methylation at a certain site of a histone can prevent DNA methylation, while histone methylation at another site can promote DNA methylation (Morselli *et al*., 2015). There are also crosstalks between different histone modifications, which can be divided into three classes: crosstalks between modifications on the same amino acid residue, on the same histone and on different histones (Murr, 2010). In addition, histone modifications can recruit chromatin remodeling complexes, but the remodeling complexes can also affect different histone modifications (Huang *et al*., 2015).

2.4 Environmental and nutritional epigenetics

The genome and the epigenome together decides the phenotype, which is the observable physical and physiological characteristics of an organism (Mazzio & Soliman, 2012). Epigenetic modifications are reversible and highly influenced by environmental factors which could contribute to the formation of phenotypes (Jaenisch & Bird, 2003). Environmental exposures can also result in inheritable phenotypic alterations that may persist through several generations (Soubry, 2015). There are many different

types of environmental exposures that can affect the epigenome and phenotype of an organism, including dietary exposures such as micronutrient deficiencies (Vanhees *et al*., 2014), low-protein diets (Carone *et al*., 2010) and high-fat diets (Wei *et al*., 2014), and life-style related exposures like smoking (Northstone *et al*., 2014), stress (Rodgers *et al*., 2013) and obesity (Soubry *et al*., 2015). Other types of environmental exposures such as pesticides (Skinner *et al*., 2013), ionizing radiation (Koturbash, 2006) and endocrine disruptors (Anway *et al*., 2006) could also influence the epigenome.

Several environmental exposures can promote abnormal phenotypes and disease states through epigenetic processes, both in the directly exposed organism and in the offspring (Guerrero-Bosagna & Skinner, 2012). Epigenetic processes can alter the expression of genes that are associated with many physiological and pathological conditions including embryonic development, aging, cancer, cardiovascular diseases, obesity, insulin resistance, type 2 diabetes mellitus, neurodegenerative diseases, reproductive defects, inflammation and immune diseases (Choi & Friso, 2010; Guerrero-Bosagna & Skinner, 2012). For example, cancer is usually associated with hypermethylation of tumor suppressor genes, as well as hypomethylation of oncogenes and in intergenic regions (Murr, 2010).

The diet is one of the main environmental factors that can affect the epigenome since food is essential for all living organisms. Both positive and negative epigenetic and phenotypic alterations can be induced by different nutrients and bioactive food compounds. An adequate intake of certain nutrients promotes and maintains healthy epigenetic patterns, while nutrient deficiencies could cause epigenetic alterations associated with diseases, as mentioned above (Mazzio & Soliman, 2014). Nutrients can alter the epigenome by changing the availability of substrates that are required in different epigenetic reactions, or by regulating the activity of the enzymes that catalyzes epigenetic modifications. Several nutrients, including methionine, folic acid, vitamin B6, vitamin B12, betaine and choline, can affect DNA and histone methylation by regulating the 1-carbon metabolism (Choi & Friso, 2010). Other food components which could influence epigenetic processes are for example niacin, biotin, pantothenic acid, genistein, green tea catechins, resveratrol, sulforaphane, curcumin, butyrate and polyunsaturated fatty acids (Burdge & Lillycrop, 2014; Choi & Friso, 2010; Vahid *et al*., 2015).

Iron has been proposed to be involved in epigenetic modifications but the specific mechanisms have not yet been explained. Many enzymes involved in DNA replication, DNA repair and epigenetic regulations, contain iron-sulfur clusters (Wessels, 2014). Furthermore, one of the demethylase families that catalyzes the demethylation of histones is jumonji C-domain-containing dioxygenases, which are iron-dependent (Chervona *et al*., 2012), and the TET enzymes that catalyze demethylation of DNA are also iron-dependent dioxygenases (Zhao & Chen, 2013). Hence, it is likely that iron deficiency affect these enzymes which might induce epigenetic changes.

The impact of zinc deficiency on the epigenome have been a bit more elucidated than the impact of iron deficiency. Zinc is an essential part of a huge number of enzymes, including many epigenetically active enzymes, such as DNA and histone methyltransferases, histone acetylases, histone deacetylases and methyl-binding proteins. Zinc is also required for the conversion of homocysteine to methionine in the 1-carbon metabolism, which affects the regeneration of S-adenosylmethionine, the methyl donor for DNA and histone methylation reactions (Wessels, 2014). Therefore, there are strong reasons to believe zinc deficiency could cause epigenetic alterations. For example, studies have shown that epigenetic modifications could explain the impaired immune defense which is frequently occurring in organisms with zinc deficiency (Wessels *et al*., 2013; Wong *et al*., 2015).

2.5 *S. cerevisiae* as a model organism for epigenetics

Efficient animal models that are suitable for research in epigenetics, are essential for establishing the role of epigenetics in relation to nutrition, disease and inheritance. Many of the epigenetic mechanisms are found both in *S. cerevisiae* and humans, which makes it possible to perform a wide range of epigenetic studies with the use of *S. cerevisiae* as a model organism. Several studies of *S. cerevisiae* have led to key discoveries of for example different chromatin remodeling complexes and enzymes that modify histones, and also the function of chromatin in gene silencing (Fuchs & Quasem, 2014; Rando & Winston, 2012). The amino acid sequence of histones are highly conserved from yeast to human, which indicates that the specific amino acid residues are important for the structure and function of the histone. A change in one single amino acid can have major consequences (Huang *et al*., 2015). One advantage with studying histones and their function in gene regulation in *S. cerevisiae* is that the yeast only has two copies of each of the core histones coding genes, while mammalian cells have 60-70 copies of each of these genes. This has made it possible to discover the function of specific histone amino acids through deletion analysis (Grunstein & Gasser, 2013).

S. cerevisiae has been the predominant model for discovering histone modifying enzymes. Studies in yeast have revealed when and where different modifications occur and how they regulate gene expression (Fuchs & Quasem, 2014). Many of the histone modifications take place at the same sites in yeast and higher eukaryotes (Morselli *et al*., 2015). Several chromatin remodeling complexes are also conserved from yeast to humans (Flaus *et al*., 2006; Swygert & Peterson, 2014). Moreover, several studies in *S. cerevisiae* have identified roles of non-coding RNAs in the regulation of chromatin structure and histone modifications (Hainer *et al*., 2011; Houseley *et al*., 2008; Thebault *et al*., 2011). However, other epigenetic mechanisms such as DNA methylation, repressive histone methylation and small interfering RNAs, do not exist in in *S. cerevisiae*. This is of course a limitation of epigenetic research in *S. cerevisiae*, but the absence of these epigenetic mechanisms also simplifies the research about other epigenetic processes like histone modifications (Fuchs & Quasem, 2014).

2.6 Growth of *S. cerevisiae* in batch cultures

S. cerevisiae can be cultured in different types of cultures such as batch, fed-batch and continuous cultures. In a batch culture, all nutrients are added from the start. No further nutrient are added during the cultivation and no medium or cells are removed. Therefore, the conditions in the culture are continually changing and the cells cannot grow indefinitely (Hogg, 2013). The growth of *S. cerevisiae* in a batch culture can be described by a growth curve, which can be divided into four different growth phases: lag phase, exponential phase, stationary phase and death phase (Figure 1) (Madigan *et al*., 2012).

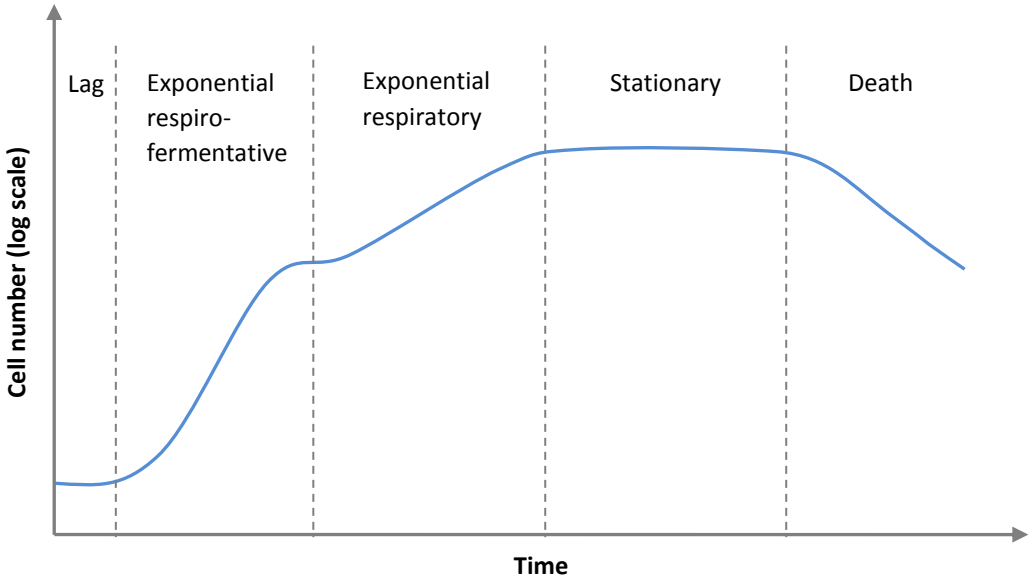

Figure 1. Growth curve and growth phases of *S. cerevisiae* cultured in a batch culture. The growth curve is shown in a semilogarithmic graph.

The lag phase is the first period of time after a cell culture is inoculated into fresh medium. In this phase, the cells adapt to the new environment before they start to grow in the exponential phase. Differences between the previous and current growth medium and the growth conditions respectively, determine the duration of the lag phase, where small differences mean a short lag phase. In the exponential phase, the cell number doubles during a constant time interval and the cells are thereby growing exponentially. Exponential growth rates can vary a lot and are affected by the culture conditions and by the characteristics of the yeast strain (Madigan *et al.*, 2012). Cell growth in the exponential phase can be described mathematically by the following expression:

$$N = N_0 e^{\mu t} \qquad (1)$$

where N is the cell number after time t, N_0 is the cell number at t=0 and μ is a constant named as the specific growth rate.

The time required for the cell number in an exponentially growing population to double is called generation time (g) (Adams & Moss, 2008) and can be expressed as:

$$g = \ln(2)/\mu \qquad (2)$$

In a glucose limited batch culture, the exponential growth phase of *S. cerevisiae* can be further divided into respiro-fermentative growth and respiratory growth as shown in Figure 1. Glucose fermentation is the dominant metabolic state during the first part of the exponential phase, but respiration on glucose also takes place to some extent. When the glucose has been used up, the cells switch to a slower, respiratory growth, where they metabolize the ethanol produced during the fermentation. There is also a short lag period when the cells switch from fermentation to respiration, due to a major change in the pattern of gene expression (Brauer *et al.*, 2005; Dickinson & Schweizer, 2004). When the glucose and the ethanol have been consumed, the exponential growth ceases and the culture enters the stationary phase. Some cells may divide and some cells may die in the stationary phase, but there is no net change in the cell number and the growth rate is zero. If the cultivation continuous further, the cells will eventually begin to die in the death phase (Madigan *et al.*, 2012).

2.7 Optical density

Measurements of turbidity, also called optical density (OD), is widely used to monitor growth of cell cultures. The more cells there are in a cell suspension, the higher the turbidity and optical density. Optical density can be measured with a spectrophotometer, which sends light of a specific wavelength through a cell suspension and measures the unscattered light that passes through. The optical density is proportional to the cell number for unicellular organisms, but within certain limits. At high cell concentrations, the optical density is no longer proportional to the cell number, since light scattered away by one cell can thereafter be scattered back by another. Measurements of optical density are easy and quick to perform and can also be made without destroying or considerably disturbing the culture. Several measurements can be made during a cultivation period and plotted versus time on a semilogarithmic graph, which can be used for calculations of the specific growth rate and generation time (Madigan *et al*., 2012). Since optical density is proportional to the cell number, Equation 1 can also be written as:

$$OD = OD_0 e^{\mu t} \quad (3)$$

where OD is the optical density after time t, OD_0 is the optical density at t=0.

Equation 3 and the specific growth rate of a culture can be obtained if an exponential regression is applied to the OD measurements of an exponentially growing culture. The generation time can subsequently be calculated using the specific growth rate according to Equation 2.

2.8 Heat stress

Heat stress causes extensive denaturation and aggregation of cellular proteins (Gabai & Sherman, 2002). The cellular response to stress conditions such as heat stress and oxidative stress, is represented by the induced gene expression of heat shock proteins (Hsps), including chaperones and proteases. Chaperones are a family of proteins that assist in the folding and refolding of various proteins, whereas proteases take part in the degradation of damaged proteins. Thus, Hsps promote cell survival by maintaining and restoring protein homeostasis. Several of the Hsps are constitutively expressed in cells but some are only expressed at stress conditions. The Hsps are also highly conserved from bacteria to humans (Arya *et al*., 2007). Expression of the genes encoding for Hsps is not only regulated by processes associated with stress but also by several other processes including the cell cycle, cell proliferation and differentiation, indicating that some Hsps have a critical role during cell growth (Hang *et al*., 1995; Jolly & Morimoto, 2000). The expression of Hsps is also regulated by several of the conditions that lead to apoptosis, including heat stress. Cells that are exposed to intense heat stress become irreparable and die, either by apoptosis or necrosis. Hsps that are induced at stress conditions are thought to function as key regulators in the control of apoptosis (Jolly & Morimoto, 2000). A recent study also showed that the genes which are important for survival of lethal heat stress, are not the same as the genes induced in response to heat stress. Identification of the genes revealed that metabolism, chromatin regulation and cellular signaling are involved in heat stress survival, but many of the genes had an unknown function (Gibney *et al*., 2013).

2.9 Oxidative stress induced by hydrogen peroxide

Oxidative stress can be caused by hydrogen peroxide (H_2O_2), which is one of the reactive oxygen species (ROS). Hydrogen peroxide is not a radical unlike other ROS and is therefore less reactive, but it can be converted to highly reactive hydroxyl radicals (•OH) in the presence of metal ions such as iron,

which is called the Fenton reaction (Valko *et al*., 2006). Hydroxyl radicals, in turn, can cause cell damage by oxidizing nuclear and mitochondrial DNA, which can lead to mutagenesis, ageing and carcinogenesis (Dizdaroglu *et al*., 2002; Marnett, 2000). Polyunsaturated fatty acids in phospholipids are also highly susceptible to oxidation by hydroxyl radicals, resulting in lipid peroxidation and damage of cell membranes. In addition, hydroxyl radicals can oxidize proteins by attacking the amino acid residues or the polypeptide backbone (Valko *et al*., 2006). In contrast to the deleterious consequences of oxidative stress, hydrogen peroxide also has beneficial roles as a signaling molecule that regulates a variety of processes including cell proliferation, differentiation and apoptosis (Bienert, 2006; Veal *et al*., 2007). The major sources of hydrogen peroxide in biological systems, is the degradation of superoxide radicals ($\bullet O_2^-$), which is a byproduct from the electron transport chain in the mitochondria. Other sources of hydrogen peroxide includes peroxisomes, phagocytic immune cells, membrane associated NADPH oxidase, cytochrome P450 metabolism and extracellular receptor-ligand interactions (Valko *et al*., 2006; Veal *et al*., 2007).

Since cells are constantly exposed to oxidative stress, they have evolved certain defense systems consisting of antioxidants and antioxidant enzymes (Jamieson, 1998; Valko *et al*., 2006). Catalase and glutathione peroxidase are the two major enzymes involved in the degradation of hydrogen peroxide and competes for hydrogen peroxide as a substrate. Elimination of hydrogen peroxide reduces the risk of forming hydroxyl radicals through the Fenton reaction which protects the cells from oxidative damage. Catalase is present in aerobic bacteria, plants and animals and is located within the peroxisomes in cells. The enzyme catalyzes the conversion of hydrogen peroxide to water and oxygen with an extremely high turnover rate. One catalase molecule can degrade about 6 million hydrogen peroxide molecules per minute (Valko *et al*., 2006). Catalase is a tetramer in which each of the four subunits contains a heme group that is bound to the catalytic site and utilized in the reduction of hydrogen peroxide (Day, 2009). High concentrations of hydrogen peroxide can lead to reduced activity or degradation of catalase, which allows for initiation of apoptosis, but an intense oxidative environment can also lead to necrosis (Cao *et al*., 2003). There are several different glutathione peroxidase isoenzymes. Many of the isoforms contain selenocysteine residues which are essential for the catalytic activity of the enzymes (Matés *et al*., 1999). The antioxidant glutathione is also important as a cofactor for glutathione peroxidase in the detoxification of hydrogen peroxide. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide to water and the oxidation of glutathione (Bhabak & Mugesh, 2010). Mitochondria are highly enriched with glutathione peroxidase since they are the major source of hydrogen peroxide in cells (Valko *et al*., 2006). The expression of glutathione peroxidase is also strongly induced by increased levels of oxidative stress caused by hydrogen peroxide (Gasch *et al*., 2000).

3 METHODS

This chapter starts with a description about the yeast strain and growth conditions used in the study, followed by the method and time schedule for development of iron and zinc deficiency in *S. cerevisiae*. The chapter also contains descriptions of the methods used for exposing *S. cerevisiae* to heat and oxidative stress and investigating the consequences of iron and zinc deficiency.

3.1 Strain, growth media and culture conditions

The yeast strain used in the study was wild type *S. cerevisiae* CBS 7764, isolated from rainbow trout intestine (Andlid *et al*., 1999). During all experiments, *S. cerevisiae* was cultured in 100 ml E-flasks

containing 30 ml defined medium developed by Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) and previously described by Albers *et al*. (1996). Ammonium sulfate (7.5 g l^{-1}) was used as nitrogen source and glucose as carbon and energy source. 2 % (w/v) glucose was used in all experiments except in the epigenetic stress experiments, where 3 % (w/v) glucose was used in order to extend the fermentative phase. Succinic acid and sodium hydroxide was used as buffer (pH 5.5) in the CBS medium. The yeast cultures were incubated at 30 °C in a rotary shaker at 190 rpm. The cultures were inoculated every 24-72 hours in new CBS medium.

During the heat and oxidative stress exposures, the cells were suspended in 0.9 % (w/v) sodium chloride (NaCl) in order to avoid cell growth that could affect the results. 30 % (w/w) hydrogen peroxide (H_2O_2) was used to expose the cells to oxidative stress. Analysis of cell survival after stress exposure was performed by spotting the cell suspensions onto YPD plates (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose and 20 g l^{-1} agar). All components for the CBS medium, sodium chloride solution and the YPD plates were autoclaved, except the vitamin solution which was sterile filtered.

3.2 Development of iron and zinc deficiency in *S. cerevisiae*

The first part of the study was to find iron and zinc concentrations that would result in deficiencies and reduced growth rates compared with control cells. This was done by culturing *S. cerevisiae* CBS 7764 in eight E- flasks with CBS medium: two control cultures with complete CBS medium (30 µM zinc and 20 µM iron); three cultures with iron limited CBS medium (0.2, 0.05 and 0.02 µM iron); and three cultures with zinc limited CBS medium (0.3, 0.1 and 0.03 µM zinc). The optical densities were measured at 600 nm (OD_{600}) regularly each day in order to monitor and compare the growth rate between the cultures. After 2 days the culture containing 0.2 µM iron and the culture containing 0.3 µM zinc were excluded. After 4 days the cultures with the lowest concentrations of iron and zinc were removed and the zinc concentration in the culture containing 0.1 µM zinc were increased to 0.3 µM. When 8 days had passed, one of the control cultures was also left out, narrowing the number of cultures down to three.

The remaining cultures were one control culture with 30 µM zinc and 20 µM iron, one culture with iron deficiency containing 0.05 µM iron and one culture with zinc deficiency containing 0.3 µM zinc. The iron concentration was lowered from 0.05 to 0.02 µM after 11 days of cultivation and further to 0.01 µM after 14 days. 32 days after the cultures were started, OD_{600} was measured once every hour for eight hours and used for calculations of the specific growth rate, µ (h^{-1}) and the generation time, g (h). The optical densities were plotted on log-scale over time and the specific growth rates were obtained from the equations for the exponential regression lines according to Equation 3. The generation times were calculated using Equation 2. During the remaining parts of the study, the cells with iron and zinc deficiency were repeatedly inoculated to new CBS medium containing 0.01 µM iron and 0.3 µM zinc respectively, and the control cells were inoculated to complete CBS medium.

3.3 Determination of optimal temperature for heat stress

When the deficiencies were evident, the optimal temperature for exposure of the cells to heat stress was determined to be able to examine the effects of the deficiencies. After 14 days of deficiency, cells from the control culture, the iron deficiency culture and the zinc deficiency culture were inoculated to OD_{600} 2 ± 0.05 in complete, iron limited and zinc limited CBS medium respectively in E-flasks. After 2.5 hours, OD_{600} was measured and cells were harvested by centrifugation at 4000 g for 5 minutes. The pellets were resuspended at OD_{600} 2 ± 0.40 in 0.9 % NaCl. OD_{600} was adjusted to 1 ± 0.30 with 0.9 %

NaCl, in order for the cellular suspensions to have the same optical density when exposed to the heat stress.

Heat stress was applied by incubating 0.5 ml of the samples at 47, 50 and 53 °C for 10 minutes in a water bath with shaking at 90 rpm. The stress was stopped by cooling the samples on ice. Analysis of viable cells was performed by making serial dilutions with 0.9 % NaCl in the range of 10^1-10^4 for each sample and spotting 10 µl drops of the 10^2-10^4 dilutions onto YPD plates in quadruplicates. The plates were incubated at 30 °C for 1-2 days and thereafter analyzed for colonies. Survival was calculated as percentage by dividing the number of colony forming units (CFU) per ml after heat stress exposure with the number of CFU/ml with non-stressed cells originating from the same cellular suspension as the stressed cells.

3.4 The effect of iron and zinc deficiency on heat stress resistance

The consequences of the deficiencies were investigated by comparing the resistance against heat stress using the temperature determined in the previous experiment. After 60 days of deficiency, cells from the control culture, the iron deficiency culture and the zinc deficiency culture were inoculated to an initial OD_{600} of 1 ± 0.05 in E-flasks with complete, iron limited and zinc limited CBS medium respectively. After 5 hours, OD_{600} was measured and cells were harvested by centrifugation at 4000 g for 5 minutes. The pellets were resuspended at OD_{600} 1.5 ± 0.30 in 0.9 % NaCl and OD_{600} was adjusted to 1 ± 0.10 with additional NaCl solution. The three cellular suspensions were subsequently diluted to 10^{-2} in two steps. Heat stress was applied by incubating 0.5 ml aliquots of the samples in duplicates at 51 °C for 5, 10 and 15 minutes in a water bath with shaking at 90 rpm. The heat stress was halted by cooling the samples on ice. The samples were diluted, spotted onto YPD plates and analyzed for colonies and survival according to the same procedure as described in section 3.3. After 4 days, the experiment was repeated using the same conditions.

3.5 Heat stress resistance and epigenetic effects of iron and zinc deficiency

To determine if the deficiencies had caused inheritable changes in the epigenome and phenotype, the cells were cultured in non-limited medium and subsequently compared for heat stress resistance. After 66 days of deficiency, yeast cells from the control culture, the iron deficiency culture and the zinc deficiency culture were inoculated to OD_{600} of 1 ± 0.10 in E-flasks with non-limited CBS medium. OD_{600} was measured every hour to determine if the cells with previous iron and zinc deficiency regained the same growth rate as the control cells. The growth rates were obtained from the exponential regression lines as described previously, and used to calculate the generation times for the three cultures. After 6 hours of cultivation in complete growth medium, when at least 2 generations had passed in all cultures, the same heat stress experiment as described in section 3.4 was performed.

24 hours after the cells were inoculated in non-limited medium, they were once again inoculated in new non-limited CBS medium. The growth rates were monitored by measuring OD_{600} once every hour for 5 hours and the generation times were calculated. Thereafter, the heat stress experiment in subchapter 3.4 was repeated to determine the duration and reversibility of possible changes in phenotype.

3.6 Determination of optimal concentration of H_2O_2 for oxidative stress

To complement the heat stress experiments and further study possible epigenetic consequences of iron and zinc deficiency, the cells were also exposed to oxidative stress by means of H_2O_2. The oxidative

stress experiments were initiated by determining the optimal concentration of H_2O_2 as follows. After 43 days of deficiency, cells from the control culture were inoculated to OD_{600} 0.95 in complete CBS medium in an E-flask. After 4.5 hours, OD_{600} was measured and cells were harvested by centrifugation at 4000 g for 5 minutes. The pellet was resuspended and adjusted to OD_{600} 1.05 in 0.9 % NaCl and subsequently diluted 10 times. Oxidative stress was applied by dividing the cellular suspension in 8 tubes and adding H_2O_2 to a concentration of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 and 1.5 M respectively and to a final volume of 5 ml for each sample. The samples were incubated at room temperature with shaking at 200 rpm for 10 minutes and then diluted immediately to stop the stress. The cellular suspensions were diluted, spotted onto YPD plates and analyzed for colonies and survival according to the same procedure as described in section 3.3.

3.7 The effect of iron and zinc deficiency on oxidative stress resistance

The effects of the deficiencies were investigated by comparing the resistance against oxidative stress using the concentrations of H_2O_2 determined in the previous experiment. After 65 days of deficiency, cells from the control culture, the iron deficiency culture and the zinc deficiency culture were inoculated to an initial OD_{600} of 1 ± 0.05 in E-flasks with complete, iron limited and zinc limited CBS medium respectively. After 5 hours, OD_{600} was measured and cells were harvested by centrifugation at 4000 g for 5 minutes. The pellets were resuspended at OD_{600} 1.5 ± 0.35 in 0.9 % NaCl and OD_{600} was adjusted to 1 ± 0.05 with additional NaCl solution. Oxidative stress was applied to duplicates of the three cellular suspensions, by adding H_2O_2 to a concentration of 0.5 M and to a final volume of 3 ml for each sample. The samples were incubated at room temperature with shaking at 200 rpm. A 100 µl sample was taken from each cellular suspension after 10, 20, 30 and 60 minutes and immediately diluted in order to stop the stress. The cellular suspensions were diluted, spotted onto YPD plates and analyzed for colonies and survival according to the same procedure as described in section 3.3.

3.8 Oxidative stress resistance and epigenetic effects of iron and zinc deficiency

To determine if the deficiencies had caused inheritable changes in the epigenome and phenotype, the cells were cultured in non-limited medium and thereafter compared for oxidative stress resistance. After 71 days of deficiency, yeast cells from the control culture, the iron deficiency culture and the zinc deficiency culture were inoculated to OD_{600} of 1 ± 0.05 in E-flasks with non-limited CBS medium. OD_{600} was measured every hour to determine if the cells with previous iron and zinc deficiency regained the same growth rate as the control cells. The growth rates and the generation times for the three cultures were calculated as before. The oxidative stress experiment described in subchapter 3.7 was repeated after 6.5 hours of cultivation in complete growth medium, when at least 2 generations had passed in each culture.

24 hours after the cells were inoculated in non-limited medium, they were once again inoculated in new non-limited CBS medium. The growth rates were monitored by measuring OD_{600} once every hour for 5 hours and the generation times were calculated. Thereafter, the oxidative stress experiment in subchapter 3.7 was repeated to study the maintenance and reversibility of possible changes in phenotype.

4 RESULTS

The following chapter presents the results from the experiments performed in the study. The outcome of the development of iron and zinc deficiency in *S. cerevisiae* is described followed by the determination of optimal conditions for the stress exposures. Subsequently, the survival after the heat and oxidative stress exposures are displayed, including the survival before and after cultivation in growth medium with non-limited iron and zinc concentrations.

4.1 Development of iron and zinc deficiency in *S. cerevisiae*

It took 14 days to find iron and zinc concentrations that resulted in markedly reduced growth rates and iron and zinc deficiency in *S. cerevisiae*. Of the three concentrations of iron (0.2, 0.05 and 0.02 µM) and zinc (0.3, 0.01, 0.03 µM) respectively that were tested first, the highest concentrations of iron and zinc were excluded after 2 days since the growth rates were not significantly different from the growth rates of the control cultures. The growth of the remaining six cultures are displayed in Figure 2 and the specific growth rate and generation time for each culture is presented in Table 1. The exponential regression lines with their equations used for calculation of the specific growth rates and generation times, can be found in Appendix A. There was only 0.1 h difference in generation time between the two control cultures and also between the cultures with zinc deficiency containing 0.1 and 0.03 µM zinc respectively (Table 1). Between the two cultures with iron deficiency containing 0.05 and 0.02 µM iron respectively, there was 0.4 h difference. The growth rate of the control cultures were approximately twice as high as the growth rate of the cultures with iron deficiency and about three times as high as the growth rate of the cultures with zinc deficiency.

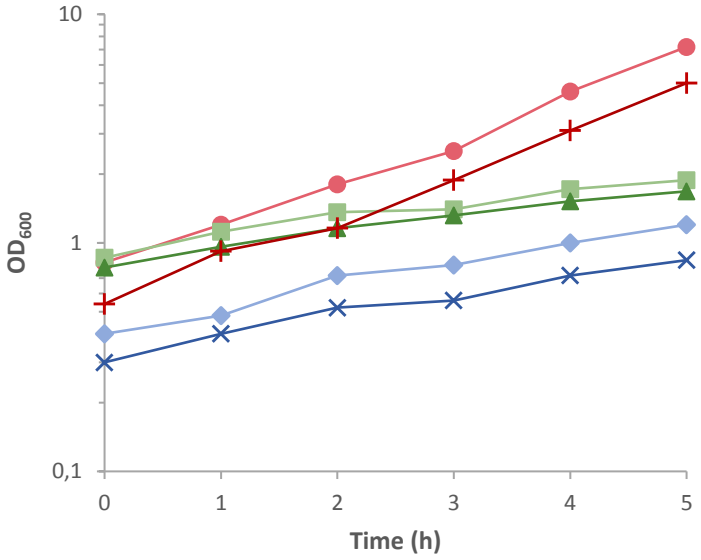

Figure 2. Growth of *S. cerevisiae* CBS 7764 after 2 days in CBS medium containing different concentrations of iron and zinc. (●,✚) control culture 1 and 2 containing 20 µM Zn and 30 µM Fe; (◆) 0.05 µM Fe; (✖) 0.02 µM Fe; (■) 0.1 µM Zn; (▲) 0.03 µM Zn.

Table 1. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 after 2 days in CBS medium containing different concentrations of iron and zinc.

Yeast culture	**Specific growth rate (h^{-1})**	**Generation time (h)**
Control 1*	0.48	1.5
Control 2*	0.49	1.4
0.05 µM Fe	0.22	3.1
0.02 µM Fe	0.20	3.5
0.1 µM Zn	0.15	4.6
0.03 µM Zn	0.15	4.5

*Containing 20 µM Zn and 30 µM Fe.

After 4 days it became evident that it takes several days for the growth rate to adjust after the iron and zinc concentrations have been changed. The growth rates of the cultures with iron and zinc deficiency continued to decrease and the cultures with the lowest iron and zinc concentrations (0.02 and 0.03 µM respectively) were therefore excluded. The zinc concentration in the remaining culture with zinc deficiency was increased from 0.1 to 0.3 µM since the cell growth had almost stopped completely. The growth rate of the iron deficiency culture was higher than the growth rate of the zinc deficiency culture and the iron concentration was therefore reduced from 0.05 to 0.02 after 11 days and further to 0.01 after 14 days. This generated a lower growth rate but it was still higher than the growth rate for the zinc deficiency culture.

After 32 days of iron and zinc deficiency, there was a clear difference in growth rate and generation time between the control culture and the deficiency cultures (Figure 3 and Table 2) but also between the two deficiency cultures. The growth rate of the control culture was 2.5 times higher than the growth rate of the iron deficiency culture and approximately seven times higher than the growth rate of the zinc deficiency culture. Exponential regression lines with equations can be found in Appendix A. After 6 hours of cultivation in CBS medium containing 2 % glucose, the growth of the control culture started to decline (Figure 3). Before the decrease in growth rate, the generation time for the control culture was 1.5 hours, which was the same as after 4 days of cultivation (Table 1 and 2). Cultivation of *S. cerevisiae* CBS 7764 at a constant iron concentration of 0.01 µM for 18 days resulted in 3.8 hours generation time and cultivation at a constant zinc concentration of 0.3 µM for 28 days generated a generation time of 10.4 hours.

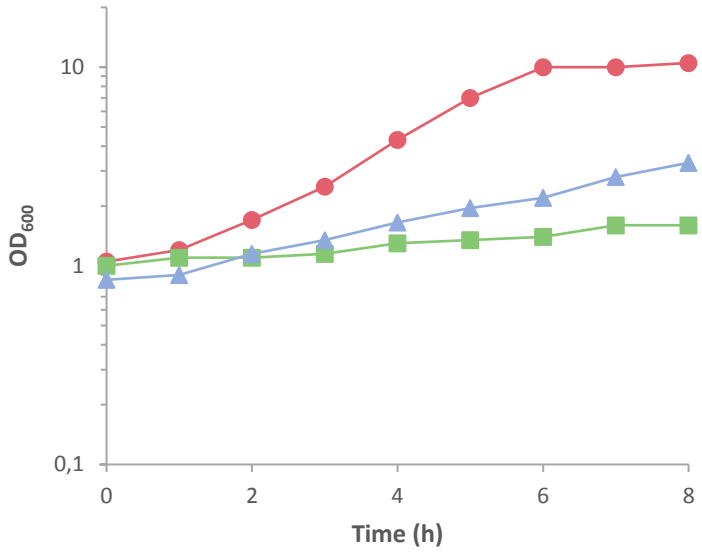

Figure 3. Growth of *S. cerevisiae* CBS 7764 in CBS medium after 32 days of iron and zinc deficiency. (●) control culture containing 20 µM Zn and 30 µM Fe; (▲) 0.01 µM Fe; (■) 0.3 µM Zn.

Table 2. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 in CBS medium after 32 days of iron and zinc deficiency.

Yeast culture	**Specific growth rate (h^{-1})**	**Generation time (h)**
Control*	0.46	1.5
0.01 µM Fe	0.18	3.8
0.3 µM Zn	0.07	10.4

*Containing 20 µM Zn and 30 µM Fe.

4.2 Determination of optimal temperature for heat stress

All data, including the number of counted colonies, the survival and the standard deviations can be found in Appendix B. The survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after 10 minutes exposure to heat stress at 47, 50 and 53 °C is visualized in Figure 4. The number of CFU/ml consisting of non-stressed cells was set to 100 % survival. At both 47 and 50 °C the survival was highest for the control cells followed by the iron deficient cells and lastly the zinc deficient cells. The survival was about the same at 47 and 50 °C for the iron and zinc deficient cells respectively. For the control sample, more colonies were counted after exposing the cells to heat stress at 47 and 50 °C than without exposing the cells to heat stress. This resulted in over 100 % survival for the control cells at these temperatures. At 53 °C the survival was approximately 0 % for the cells from all three cultures. The optimal temperature for heat stress to be used in the following experiments for investigation of the effects of iron and zinc deficiency was determined to 51 °C.

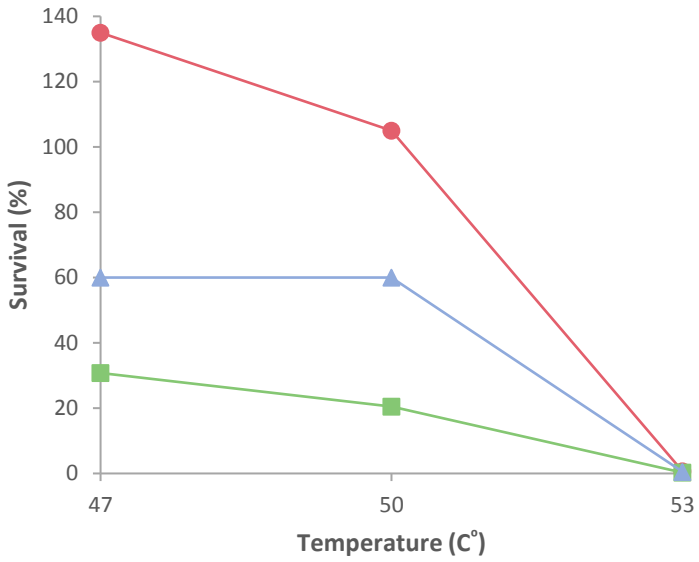

Figure 4. Survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after 10 minutes of heat stress exposure at three different temperatures. (●) control cells; (▲) iron deficient cells; (■) zinc deficient cells.

4.3 The effect of iron and zinc deficiency on heat stress resistance

All data, including the number of counted colonies, the survival and the standard deviations can be found in Appendix B. The result from the investigation of the heat stress resistance of *S. cerevisiae* CBS 7764 after 60 days of iron and zinc deficiency is presented in Figure 5. Similar survival curves were obtained for the control and the iron deficient cells, but the zinc deficient cells were less resistant against heat stress than the control and the iron deficient cells. After 5 minutes exposure to heat stress at 51 °C, the survival of the zinc deficient cells was only 5 % while the survival for the control and iron deficient cells was about 80 %. After 10 minutes, the survival was less than 5 % for both the control cells and the deficient cells and after 15 minutes the survival was approximately 0 %.

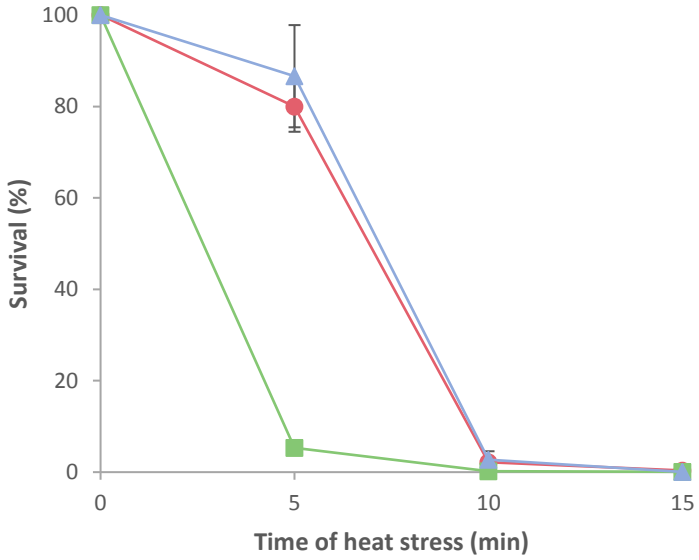

Figure 5. Survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. (●) control cells; (▲) iron deficient cells; (■) zinc deficient cells. The results represent mean values of two independent experiments and error bars are presented as standard deviations.

4.4 Heat stress resistance and epigenetic effects of iron and zinc deficiency

The growth of *S. cerevisiae* CBS 77764, when the CBS medium was restored to non-limited iron and zinc concentrations after 66 days of iron and zinc deficiency, is displayed in Figure 6. The specific growth rates and the generation times are presented in Table 3 and the exponential regression lines including equations can be found in Appendix A. After 2 hours of cultivation, the previously iron deficient cells almost reached the same growth rate as the control cells and there was only 0.1 h difference in generation time between the two cultures. The generation time of the previously iron deficient cells had thereby decreased from 3.8 to 1.6 hours (Table 2 and 3). It took 4 hours for the previously zinc deficient cells to gain a growth rate which was only slightly lower than the growth rate for the control cells (Figure 6). The generation time had then decreased from 10.4 to 1.8 hours, which was only 0.3 hours longer than the generation time for the control cells (Table 2 and 3).

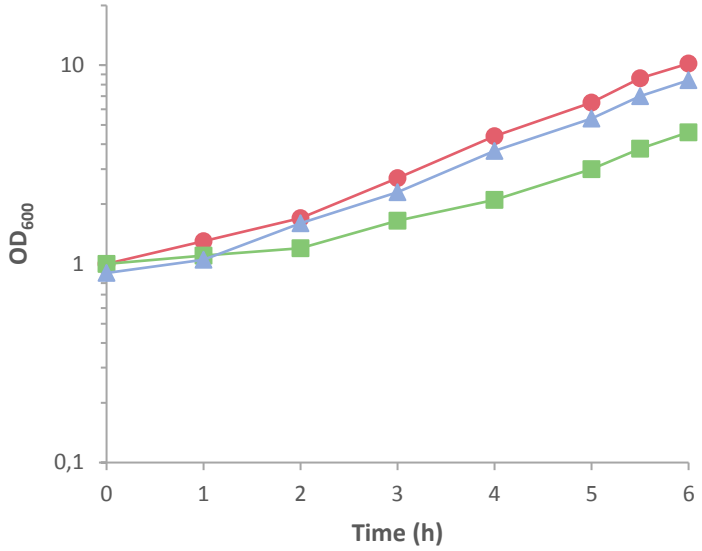

Figure 6. Growth of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 3. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc.

Yeast culture	**Specific growth rate (h^{-1})**	**Generation time (h)**
Control	0.45	1.5
Previously iron deficient	0.42	1.6
Previously zinc deficient	0.39	1.8

The cells were exposed to heat stress after 6 hours of cultivation in non-limited CBS medium when 3.3 generations had passed in the control culture, 3.1 generations in the culture with former iron deficiency and 2.2 generations in the culture with former zinc deficiency. All data, including the number of counted colonies, the survival and the standard deviations can be found in Appendix B. Figure 7 shows the survival of *S. cerevisiae* CBS 7764 after heat stress exposure at 51 °C, for investigation of possible changes in phenotype and the epigenome caused by iron and zinc deficiency. The control cells and the previously iron deficient cells followed similar survival curves while the previously zinc deficient cells were still more sensitive against heat stress. After 5 minutes of heat stress exposure

there was no decrease in survival for the control cells and the formerly iron deficient cells while the survival for the previously zinc deficient cells were about 70 %. After 10 minutes the survival was only 6 % for the control cells and approximately 0 % for the previously deficient cells.

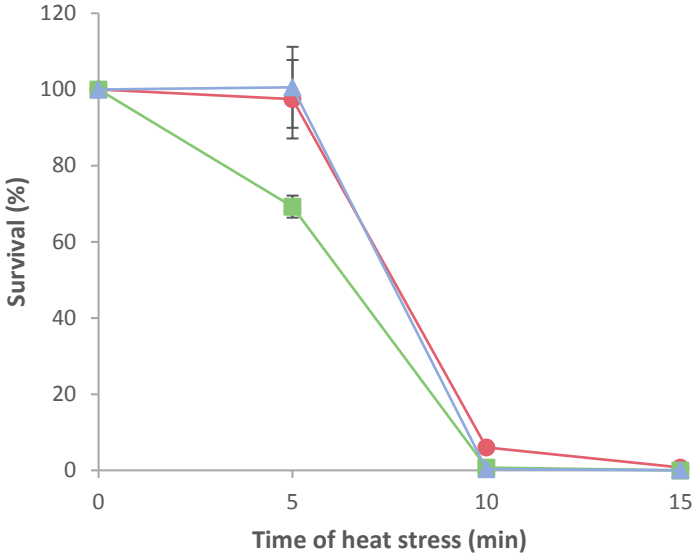

Figure 7. Survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. The cells were cultivated in non-limited CBS medium for 6 hours before they were exposed to heat stress. (●) control cells; (▲) previously iron deficient cells; (■) previously zinc deficient cells. Error bars are presented as standard deviations.

The growth of *S. cerevisiae* CBS 7764 24 hours after the CBS medium was restored to non-limited iron and zinc concentrations are displayed in Figure 8 and the specific growth rate and generation time for the three cultures are presented in Table 4. Exponential regression lines and equations for the growth rate can be found in Appendix A. There was no significant difference in growth rate and generation time between any of the cultures. The generation time was 1.6 hours for both the control culture and the culture with former iron deficiency and the generation time for the culture with previous zinc deficiency was only 0.1 hours longer. There was also no significant difference between the specific growth rate and the generation time obtained at this time and 24 hours earlier.

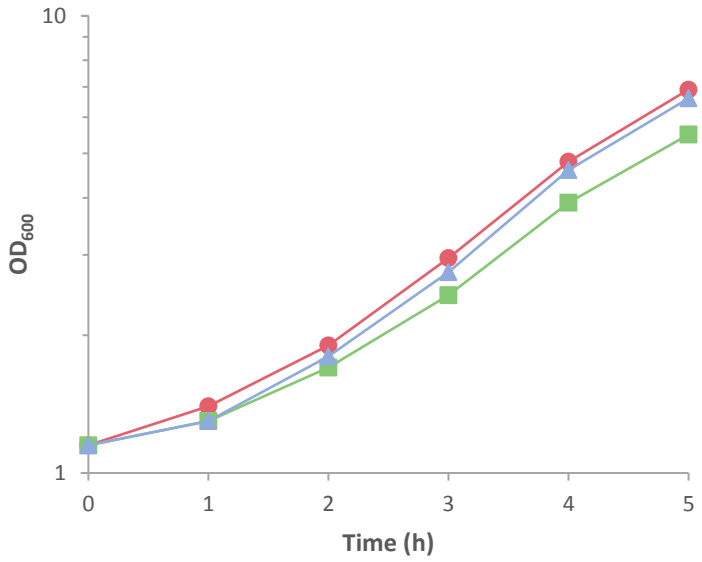

Figure 8. Growth of *S. cerevisiae* CBS 7764 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 4. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc.

Yeast culture	**Specific growth rate (h^{-1})**	**Generation time (h)**
Control	0.44	1.6
Previously iron deficient	0.44	1.6
Previously zinc deficient	0.40	1.7

Figure 9 shows the heat stress resistance of *S. cerevisiae* CBS 7764 with former zinc and iron deficiency after 29 hours of cultivation in non-limited growth medium when approximately 17 generations had passed in all cultures. All data from the heat stress experiment can be found in Appendix B. The previously zinc deficient cells were most resistant against the heat stress followed by the control cells and lastly the previously iron deficient cells. After 5 minutes exposure to heat stress at 51 °C, the survival was approximately 50 % for the control cells, 35 % for the previously iron deficient cells and 70 % for the previously zinc deficient cells. The survival was approximately 0 % for the cells from all three cultures after 10 minutes of heat stress.

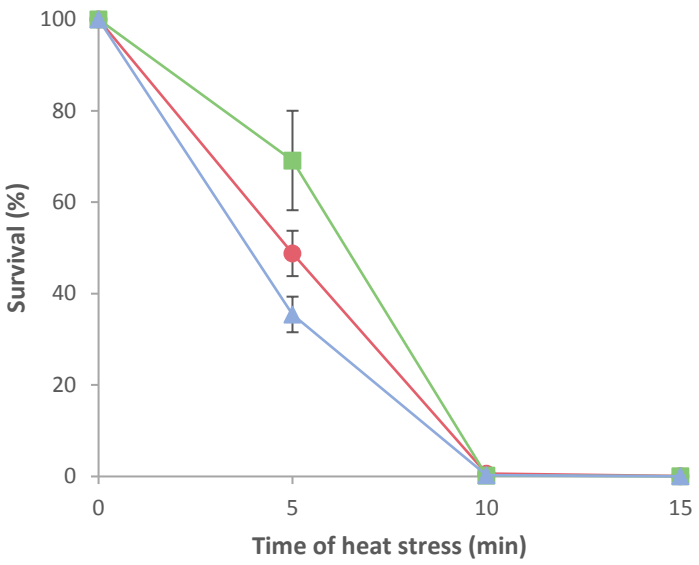

Figure 9. Survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. The cells were cultivated in non-limited CBS medium for 29 hours before they were exposed to heat stress. (●) control cells; (▲) previously iron deficient cells; (■) previously zinc deficient cells. Error bars are presented as standard deviations.

4.5 Determination of optimal concentration of H_2O_2 for oxidative stress

All data, including the number of counted colonies, the survival and the standard deviations can be found in Appendix B. The survival of *S. cerevisiae* CBS 7764 after 10 minutes exposure to oxidative stress by means of H_2O_2 at concentration up to 1.5 M is visualized in Figure 10. The survival decreased with increasing concentration of H_2O_2 all the way up to 0.8 M when the survival was about 0 %. The optimal concentration of H_2O_2 for oxidative stress to be used in the following experiments for investigation of the effects of iron and zinc deficiency was determined to 0.5 M.

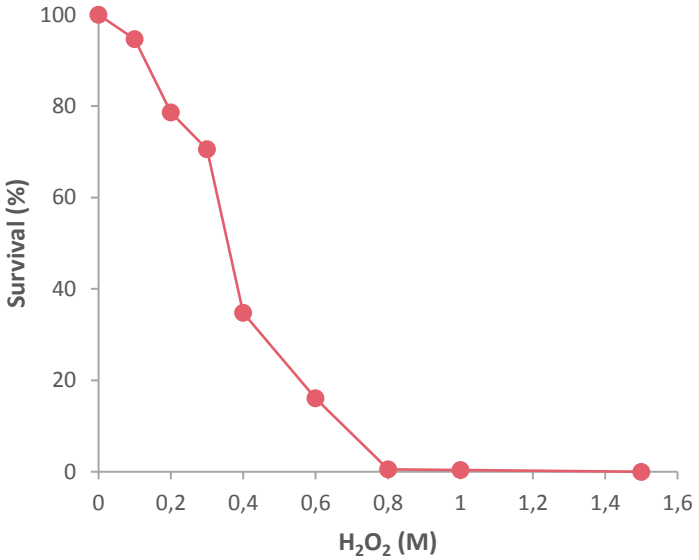

Figure 10. Survival of *S. cerevisiae* CBS 7764 after 10 minutes exposure to H_2O_2 at concentrations up to 1.5 M. The cells were cultivated in non-limited CBS medium.

4.6 The effect of iron and zinc deficiency on oxidative stress resistance

The result from the investigation of the oxidative stress resistance of *S. cerevisiae* CBS 7764 after 65 days of iron and zinc deficiency is presented in Figure 11. The data from the oxidative stress experiment can be found in Appendix B. Overall, the iron and zinc deficient cells were less resistant against oxidative stress than the control cells and survived for a shorter period of time. After 10 minutes exposure to 0.5 M H_2O_2, the survival was about 55 % for the control cells and the iron deficient cells, and 30 % for the iron deficient cells although the standard deviation was large for the iron deficient cells. The survival decreased to approximately 40 % for the control cells after 20 minutes of oxidative stress exposure and to about 5 % for the deficient cells. After 30 minutes, the survival of the control cells decreased to 17 % and further to about 2 % after 60 minutes of oxidative stress exposure. None of the deficient cells survived after 30 minutes of stress.

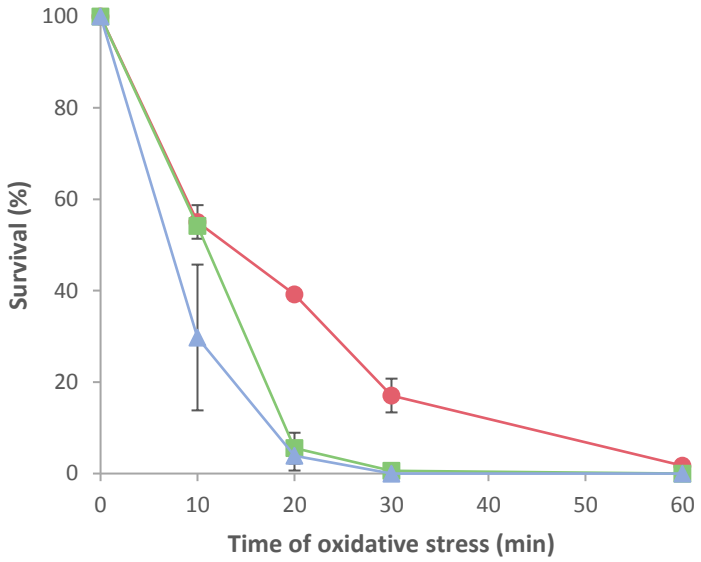

Figure 11. Survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes. (●) control cells; (▲) iron deficient cells; (■) zinc deficient cells. Error bars are presented as standard deviations.

4.7 Oxidative stress resistance and epigenetic effects of iron and zinc deficiency

The growth of *S. cerevisiae* CBS 7764 when the CBS medium was restored to non-limited iron and zinc concentrations after 66 days of iron and zinc deficiency, is displayed in Figure 12. The specific growth rates and the generation times are presented in Table 5 and the exponential regression lines including equations can be found in Appendix A. After 2 hours of cultivation, the previously iron deficient cells reached the same growth rate and generation time as the control cells. The generation time of the previously iron deficient cells had thereby decreased from 3.8 to 1.7 hours (Table 2 and 5). It took 3 hours for the previously zinc deficient cells to gain a growth rate which were slightly lower than the growth rate for the control cells (Figure 12). This resulted in a decrease in the generation time from 10.4 to 2.1 hours, which was only 0.4 hours longer than the generation time for the control cells (Table 2 and 5).

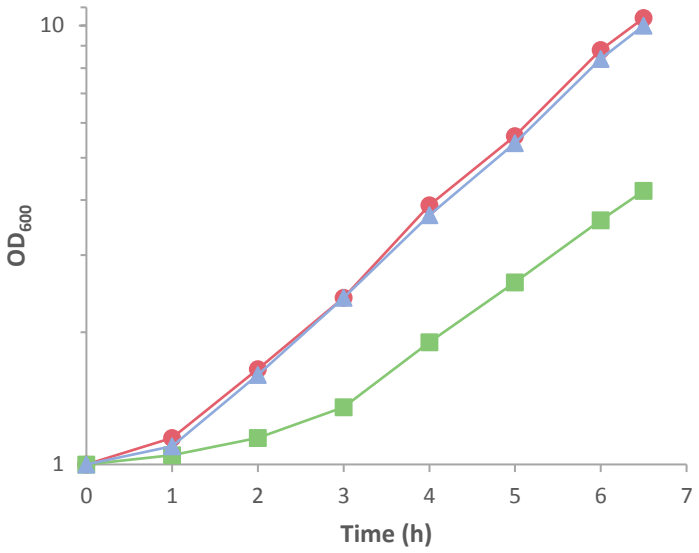

Figure 12. Growth of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 5. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc.

Yeast culture	**Specific growth rate (h^{-1})**	**Generation time (h)**
Control	0.41	1.7
Previously iron deficient	0.41	1.7
Previously zinc deficient	0.32	2.1

The cells were exposed to oxidative stress after 6.5 hours of cultivation in non-limited CBS medium when 3.3 generations had passed in the control culture, 3.3 generations in the culture with former iron deficiency and 2.1 generations in the culture with former zinc deficiency. All data from the stress experiment can be found in Appendix B. Figure 13 shows the survival of *S. cerevisiae* CBS 7764 after exposure to 0.5 M H_2O_2, for investigation of possible changes in phenotype and the epigenome caused by iron and zinc deficiency. The deficient cells were more sensitive to oxidative stress than the control cells and had a high killing rate during the first 10 minutes of stress exposure. After 10 minutes, the survival was approximately 70 % for the control cells, 20 % for the previously iron deficient cells and 37 % for the previously zinc deficient cells. The survival decreased further after 20 and 30 minutes, with a similar killing rate for both the control and deficient cells. After 60 minutes of oxidative stress, the survival was approximately 0 % for the cells from all three cultures.

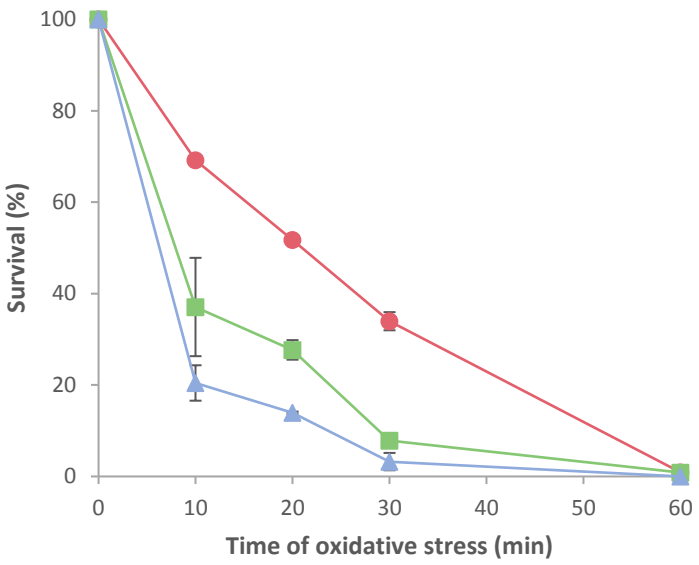

Figure 13. Survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes. The cells were cultivated in non-limited CBS medium for 6.5 hours before they were exposed to oxidative stress. (●) control cells; (▲) previously iron deficient cells; (■) previously zinc deficient cells. Error bars are presented as standard deviations.

The growth of *S. cerevisiae* CBS 7764 24 hours after the CBS medium was restored to non-limited iron and zinc concentrations are displayed in Figure 14 and the specific growth rate and generation time for the three cultures are presented in Table 6. Exponential regression lines and equations for the growth rate can be found in Appendix A. There was no significant difference in growth rate and generation time between any of the cultures. The generation time was 1.7 hours for both the control culture and the culture with former iron deficiency and the generation time for the culture with previous zinc deficiency was only 0.2 hours longer. There was also no significant difference between the specific growth rate and the generation time obtained at this time and 24 hours earlier.

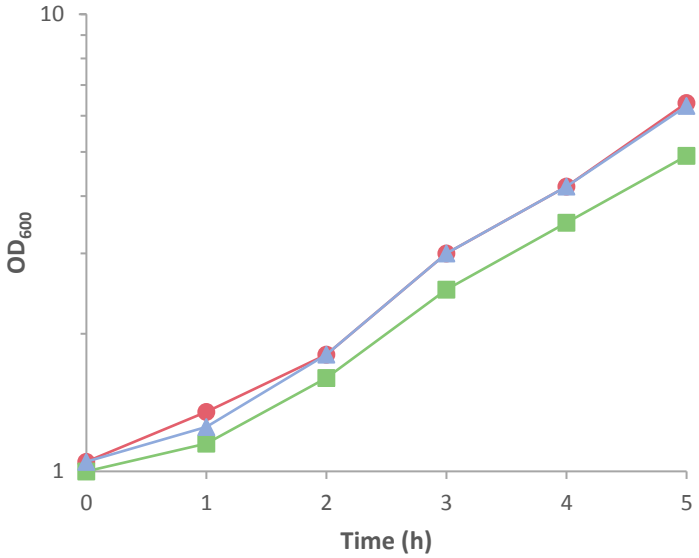

Figure 14. Growth of *S. cerevisiae* CBS 7764 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 6. Specific growth rate and generation time of *S. cerevisiae* CBS 7764 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc.

Yeast culture	Specific growth rate (h^{-1})	Generation time (h)
Control	0.41	1.7
Previously iron deficient	0.41	1.7
Previously zinc deficient	0.37	1.9

Figure 15 shows the oxidative stress resistance of *S. cerevisiae* CBS 7764 with former zinc and iron deficiency after 29 hours of cultivation in non-limited growth medium when approximately 15 generations had passed in all cultures. All data from the experiment can be found in Appendix B. The survival curves were similar for both the control cells and the cells with previous iron and zinc deficiency and there was no significant difference in survival at any time. After 10 minutes exposure to H_2O_2, the survival was approximately 70 % for the cells from all cultures. The survival decreased to about 60 % after 20 minutes of stress and further to about 50 % after 30 minutes. Only 5 % of the cells survived after 60 minutes of oxidative stress.

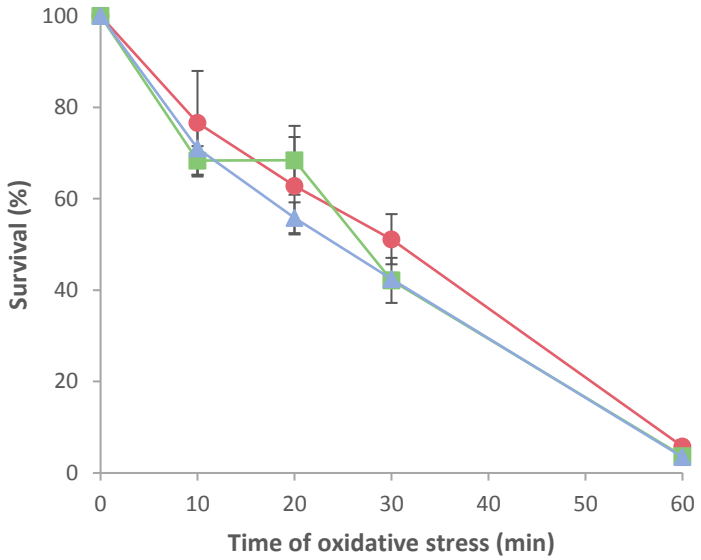

Figure 15. Survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes. The cells were cultivated in non-limited CBS medium for 29 hours before they were exposed to oxidative stress. (●) control cells; (▲) previously iron deficient cells; (■) previously zinc deficient cells. Error bars are presented as standard deviations.

5 DISCUSSION

This chapter includes a discussion about the methods and the results from the development of iron and zinc deficiency in *S. cerevisiae* and from the different heat and oxidative stress experiments. The chapter also contains a discussion about the research questions stated in section 1.1, including the use of *S. cerevisiae* as a model organism for nutritional epigenetics.

5.1 Development of iron and zinc deficiency in *S. cerevisiae*

When the iron and zinc deficiencies were developed in *S. cerevisiae* it became clear that it takes several days for the cells to adjust to the new iron and zinc concentrations, and for the growth rate to become stable in the early exponential phase. Therefore, the zinc concentration had to be increased in the zinc deficiency culture after 4 days since the growth almost stopped completely. It can also be seen that it

takes time for the cells to adjust to the new concentrations, when comparing the growth rates and generation times for the zinc deficiency cultures after 2 days and after 32 days of cultivation (Table 1 and 2). The growth rate was lower and the generation more than twice as long after 32 days compared to after 2 days, even though the zinc concentration was higher after 32 days. Because of this time required for adaptation, it took several weeks to find iron and zinc concentrations that resulted in highly reduced growth rates. Also, since the cultivation was performed in batches, the conditions were continually changing and the iron and zinc concentrations declined until the cells were inoculated in new medium. Therefore, the cells did not adjust to specific concentrations of iron and zinc, but rather to concentrations below the ones in the fresh CBS medium.

The final concentrations chosen for the deficiency cultures, that showed markedly reduced growth rates, were 0.01 µM iron and 0.3 µM zinc. This corresponds to a 2000 times reduction of the iron concentration and a 100 times reduction of the zinc concentration compared to the control medium which contained 20 µM iron and 30 µM zinc. But the growth rate was still significantly lower for the zinc deficiency culture than for the iron deficiency culture (Figure 3 and Table 2), which indicates that zinc might be more essential than iron for growth of *S. cerevisiae* during fermentation. Iron is important for many proteins involved in the electron transport chain, which is utilized during respiration but not fermentation, while zinc is an important part of many different proteins including zinc finger proteins involved in transcription, as mentioned in section 2.1 and 2.2. This could be an explanation for the difference in growth rate between the iron and zinc deficient cells. The reason behind the 2000 and 100 times reduction of the iron and zinc concentration respectively, could also be that the non-limited CBS medium might contain more iron than what is required for non-limited growth of *S. cerevisiae*. It would have been preferable to reduce the iron concentration to an even lower level than 0.01 µM in the iron deficiency culture, in order to obtain a lower growth rate and further promote epigenetic changes, and also to have a growth rate more similar to the zinc deficiency culture, but this was not possible due to the time limit of the project.

The generation time of *S. cerevisiae* CBS 7764 cultivated in non-limited CBS medium was determined several times for the same culture. All of the calculated generation times were between 1.4 and 1.7 hours (Table 1-6), which was very close to the generation time for the same yeast strain determined to 1.6 hours by Blomberg (1997). This indicates that the cell cultures were growing properly and that the growth of the cultures with iron and zinc deficiency were not limited by anything else than the lack of iron and zinc.

5.2 Methods for heat and oxidative stress experiments

There are several parts of the methods used for the heat and oxidative stress experiments that could have affected the results of all or several of the stress experiments. Firstly, it is well documented that the stress resistance of *S. cerevisiae* varies between the different growth phases in a batch culture. Respiring cells growing in the late exponential or stationary phase are much more resistant against a number of different stresses, including heat and oxidative stress, than cells growing in the early exponential phase (Dickinson & Schweizer, 2004; Jamieson, 1998; Kronberg *et al*., 2008). Therefore, it is important to consider how much time that passes after the cells have been inoculated in fresh medium until they are exposed to the stress. This time period was 5 hours in all stress experiments in this study, except in the first heat stress experiment for determination of the optimal temperature where it was 2.5 hours, and in the first epigenetic heat and oxidative stress experiment where it was 6 and 6.5 hours respectively.

The survival at 51 °C after 10 minutes in the first heat stress experiment (Figure 4) was higher than the survival after 10 minutes in the other heat stress experiments (Figure 5, 7 and 9). This difference might be due to that the cells from the first heat stress test still contained some protecting molecules or processes from the respiratory phase before the inoculation. The survival curves from the heat stress experiments (Figure 5 and 7) show a heavy decrease in survival between 0 and 5 minutes or between 5 and 10 minutes. This large decrease in survival between two times could probably have been avoided if samples had been taken at a shorter time interval than 5 minutes.

To investigate if possible epigenetic effects remained at least 2 generations after the medium had been restored to non-limited iron and zinc concentrations, the cells had to be cultured for 6 and 6.5 hours before the first epigenetic heat and oxidative stress experiment respectively (Figure 6 and 12). In these experiments, the glucose concentration in the CBS medium was increased from 2 to 3 % to prevent the cells from entering the respiratory phase. The reason behind the increase was the reduced growth rate of the control culture after 6 hours (Figure 3), which probably was caused by the switch from fermentation to respiration in the cells.

The cell suspensions used in the stress experiments were not homogeneous, which in combination with all the dilutions in the experimental procedures, contributed to variations in the results. This can be seen when comparing the number of counted colonies in the four different spots on the YPD plates, that all came from the same sample (Appendix B). For many samples, the number of colonies was very similar in the four spots, but there was also around 20 colonies difference between some spots. In plate counting it is also assumed that each colony originates from one single cell, which might not always be the case. Also, the stressed cells grew much slower than the non-stressed cells that served as references for 100 % survival. Therefore, some of the colonies might have been missed when the colonies were counted, especially the non-stressed. During the oxidative stress tests, a lot of bubbles were formed in the samples most likely from the degradation of hydrogen peroxide to water and oxygen. It was difficult to take out samples without including any bubbles, which might have affected the sample volumes and hence resulted in fewer colonies on the plates. All these factors mentioned above could have affected the results from the stress experiments and the standard deviations of the survival. The size of the standard deviations differed quite a lot between different samples and experiments. Therefore it is not possible to say that a specific temperature or concentration of hydrogen peroxide results in a specific survival, but it is possible to discover trends. Of course the experiments performed in this study need to be repeated in order to verify the results. It would be better to have at least two cultures with each type of deficiency and also to increase the number of spots used when the colonies are counted on the YPD plates.

A major issue related to the oxidative stress experiments was how to stop the stress in order to limit the oxidative stress exposure to a certain time range. It was concluded that 1000 times dilution was required to stop the stress, and the 10^2 dilutions that were spotted onto the YPD plates were therefore not utilized when the colonies were counted in the oxidative stress experiments. An alternative could be to include the hydrogen peroxide in the YPD medium in the plates, but then the cells needs longer time to grow, which would not have been possible in this study. It might also be possible to centrifuge the samples and remove a certain volume of the supernatant and replace this with 0.9 % NaCl, instead of pouring of the supernatant which might affect the dilution.

The survival after 10 minutes exposure to 0.5 M hydrogen peroxide in the first oxidative stress experiment where the optimal hydrogen concentration was determined (Figure 10) was lower than

the survival after 10 minutes in the other oxidative stress experiments (Figure 11, 13 and 15). This was probably because the samples in the first oxidative stress experiment were diluted 10 times before the stress test and the hydrogen peroxide was thereby diluted 100 times instead of 1000 times after the stress exposure. The cells in the first oxidative stress experiment were thereby affected by the hydrogen peroxide on the YPD plates, which resulted in a lower survival. Only single samples were used in the first oxidative stress experiment, which implies that these results are less reliable. This could also have contributed to the difference in survival after 10 minutes between the experiments.

One issue with the epigenetic stress experiments is that the mother cells, which have been directly exposed to iron and zinc deficiency, take part in the stress exposures in addition to the daughter cells, which have not been exposed to iron and zinc deficiency. This is not optimal for studying inheritance of epigenetic modifications since the mother cells also contribute to changes in stress tolerance and phenotype. But when 2 generations have passed in the cultures, only 25 % of the cells are mother cells that have been directly exposed to the deficiencies, which means that 75 % of the phenotypic changes are still caused by inheritable epigenetic changes.

5.3 Determination of optimal stress conditions

In the first heat stress experiment, the optimal temperature for heat stress was determined to 51 °C. The aim was to have a temperature that also affected the control cells, since an intense heat stress might reveal differences in heat resistance more easily and hence changes in phenotype. It could thereby be concluded from the survival in Figure 4 that the optimal temperature was between 50 and 53 °C. A temperature of 52 °C resulted in a too low survival (not included in this thesis) and 51 °C was subsequently chosen as the optimal temperature. The first heat stress experiment also showed that the zinc deficient cells was less resistant against heat stress than the iron deficient cells (Figure 4), which was expected since the zinc deficient cells grew much slower than the iron deficient cells (Table 2). The survival of the control cells in the first heat stress experiment was 135 % at 47 °C (Figure 4). This high survival was probably due to that a dilution (10^4) with fewer colonies were used when the colonies were counted compared to the other experiments (Appendix B), which means that the statistical significance were lower. Also, only single samples were used in this stress experiment instead of duplicates as in the other experiments. Other experimental factors as the ones discussed above, including the heterogeneity of the cell suspensions, could also have contributed to the high survival.

The optimal concentration of hydrogen peroxide for oxidative stress was determined to 0.5 M. As for the first heat stress test, the aim was to generate an intense oxidative stress that also would affect the control cells. This experiment was though performed only with the control cells in order to save time. 0.4 M hydrogen peroxide was tried after the first oxidative stress experiment but resulted in too high survival (not included in this thesis) after which 0.5 M was tried and chosen as the optimal concentration of hydrogen peroxide. Since the heat and oxidative stress tolerance varies between different *S. cerevisiae* strains (Çakar *et al*., 2005; Morano *et al*., 2012) and there is no more data on stress tolerance for *S. cerevisiae* CBS 7764, it is not possible to compare the levels of stress resistance from this study with other studies.

5.4 The effect of iron and zinc deficiency on heat and oxidative stress resistance

Iron and zinc have several essential functions in the cell as described in section 2.1 and 2.2, which implies that iron and zinc deficiency causes reduced resistance against different types of stress. The

experiments in this study showed a reduced heat stress tolerance for the cells with zinc deficiency, and a reduced oxidative stress tolerance both for the cells with iron deficiency and for the cells with zinc deficiency (Figure 5 and 11). However, the cells with iron deficiency did not show a reduced tolerance against heat stress compared to the control cells (Figure 5), which might be because the iron concentration and the growth rate were not low enough. But this was probably not the case since the iron deficient cells showed a reduced heat stress resistance in the first heat stress experiment where the optimal temperature was decided. A reduced heat stress tolerance was also shown for the iron deficient cells in three other heat stress experiments performed in the beginning of the study (not included in this thesis), when the methods were developed and the stress experiments were performed about 16 hours after inoculation instead of 5 hours. The increase in heat tolerance for the iron deficient cells after demonstrating a low heat tolerance in several experiment, might be caused by a mutation or an epigenetic change induced by the iron deficiency. This change also hindered the following heat stress experiments that were supposed to investigate possible epigenetic effects of iron deficiency.

The iron deficient cells were slightly less resistant to the oxidative stress than the zinc deficient cells, as can be seen in Figure 11. The same trend was also shown in several other oxidative stress experiments, which are not included in this thesis. This might seem contradictive since the zinc deficient cells were growing much slower than the iron deficient cells and also because there is less iron that could catalyze the Fenton reaction, which would mean a lower production of hydroxyl radicals and thereby less oxidative damage. But other transition metals such as chromium, cobalt and copper, can also catalyze the Fenton reaction (Jomova & Valko, 2011) and iron is also essential for the enzyme catalase, which degrades hydrogen peroxide as described in section 2.9. Consequently, the iron deficient cells might not be capable of degrading the hydrogen peroxide to the same extent, which could be an explanation for the low oxidative stress resistance of the iron deficient cells.

5.5 Epigenetic effects of iron and zinc deficiency

Figure 6 and 12 and Table 3 and 5 show that the previously deficient cells regained almost the same growth rate as the control cells after only a few hours. The purpose of restoring the growth rates before performing the epigenetic stress experiments was to exclude the impact of direct effects of the deficiencies on the stress resistance. But epigenetic modifications might also result in a lower growth rate, which means that differences in stress tolerance between control cells and previously deficient cells may still be caused by epigenetic modifications even if the growth rate is not restored. There was a slight difference in growth rate between the control cells (0.41 h^{-1}) and the previously zinc deficient cells (0.32 h^{-1}) before the first epigenetic oxidative stress experiment (Table 5), but the difference was still very small compared to earlier when the growth rate of the zinc deficient cells was 0.07 h^{-1} (Table 2) and therefore the growth rate was considered as restored. However, it is possible that a small part of the reduced oxidative stress resistance of the previously zinc deficient cells in Figure 13 was caused by direct effects of the zinc deficiency. The growth rates of the previously iron and zinc deficient cells, 24 hours after the CBS medium was restored to non-limited iron and zinc concentrations, was even more similar to the growth rate of the control cells, which further indicates that the growth rates were completely restored (Figure 8 and 14; Table 4 and 6).

The stress experiments were used as a method to discover changes in the phenotype of *S. cerevisiae* CBS 7764, which might be caused by epigenetic modifications. A lower stress tolerance for the previously deficient cells compared to the control cells means that the phenotype has changed. The

first epigenetic heat stress experiment revealed a phenotypic change in the previously zinc deficient cells, that had been inherited for two generations (Figure 7). Although, the difference in heat resistance between the control cells and the previously zinc deficient cells was a lot smaller in the epigenetic heat stress experiment compared to the earlier heat stress test shown in Figure 5. But an explanation for this may be that the highly reduced heat stress resistance in Figure 5 was caused by a combination of direct and epigenetic effects, while the more slightly reduced stress tolerance in Figure 7 was caused only by epigenetic effects.

Epigenetic modifications are reversible, as mentioned earlier, and the reversibility of the modifications was used in this study to exclude that the phenotypic changes were caused by genetic mutations. After 29 hours of cultivation in non-limited medium, the second epigenetic heat stress test was performed in order to investigate the reversibility of the phenotypic change. The experiment showed a higher stress tolerance for the previously zinc deficient cells compared to the control cells and the previously iron deficient cells (Figure 9). If the reduced heat stress tolerance for the previously zinc deficient cells in Figure 7 had been reversed, the survival curves of the control cells and the former zinc deficient cells in Figure 9 should have been the same, and also similar to the survival curves of the control cells in the earlier heat stress experiments (Figure 5 and 7). The result from the second heat stress experiment (Figure 9) was probably due to experimental errors. This makes it hard to draw any conclusion from the experiment and therefore it was not possible to determine the reversibility of the phenotypic change shown in the first epigenetic heat stress experiment (Figure 7). It is possible though, that new reversible phenotypes caused by epigenetic modifications could have been revealed if there had been time to repeat the heat stress experiments. This could have been true also for the iron deficient cells that did not show a reduced heat stress resistance in this study as discussed in section 5.4.

The result from the first epigenetic oxidative stress experiment, visualized in Figure 13, shows an altered phenotype both for the previously iron deficient and the zinc deficient cells, which had been inherited for three and two generations respectively. Similar survival curves for the cells in Figure 11 and 13 further increases the credibility of these result and excludes experimental errors. The second epigenetic oxidative stress experiment showed no difference in oxidative stress tolerance between the control cells and the previously iron and zinc deficient cells, including many overlapping error bars (Figure 15). This indicated that the phenotypic changes had been reversed. All three survival curves in the second oxidative stress experiment (Figure 15) were also very similar to the survival curve of the control cells in the earlier oxidative stress experiments (Figure 11 and 13), which indicates that the results from the oxidative stress experiments are reliable.

The phenotypic changes observed in the heat and oxidative stress experiments could have been caused by epigenetic changes at specific genes involved in heat and oxidative stress responses. But there are also general stress responses which answers to several different stresses (Morano *et al*., 2012), which means that the phenotypic changes could have been a consequence of epigenetic modifications at genes involved in the general stress response as well. Also, as mentioned in section 2.8, it might not be the stress induced genes that are most important for survival. Therefore, epigenetic modifications at other important genes, not involved in the stress response, could have been the reason for the phenotypic changes.

Because of the time limit this project, it was not possible to, on a molecular level, confirm that the phenotypic changes observed in the experiments were caused by epigenetic modifications. The method used in this study does not reveal which type of epigenetic modification that has occurred or

at which location, but the method can still be useful for detecting compounds that could affect the epigenome and therefore are relevant for further studies. Mass spectrometry or chromatin immunoprecipitation techniques, which utilizes antibodies that recognizes specific histone modifications, could be used in further studies in order to identify and locate histone modifications (Huang *et al*., 2015). Since epigenetic alterations affect the gene expression, it is also highly relevant to analyze the gene expression, for example by using RNA-sequencing (Miao *et al*., 2013), in order to relate epigenetically induced gene expressions to changes in phenotype. It could also be relevant to sequence the DNA when a phenotypic change has been discovered, in order to exclude mutations.

5.6 *S. cerevisiae* as a model organism for nutritional epigenetics

As mentioned in section 2.5, it is essential to have efficient model organisms that can be used to study the relation between epigenetics, health and disease. One of the purposes with this study was to evaluate if *S. cerevisiae* can be used as an efficient model organism for studies in nutritional epigenetics. *S. cerevisiae* is one of the most utilized and well-studied model organisms overall, and therefore the knowledge about the yeast is huge. General advantages is that *S. cerevisiae* is robust, cheap, easy to culture and has a short life cycle, which for example reduces the time required to perform experiments. *S. cerevisiae* can easily be genetically manipulated and a lot of genetic tools are available, which makes it possible to perform many different types of experiments.

One of the major disadvantages with epigenetic studies in *S. cerevisiae* is that the yeast lacks DNA methylation, but this epigenetic mechanism could instead be investigated in other model organisms such as Caco-2 cells (Yara *et al*., 2013). But the histone proteins and many epigenetically active enzymes are conserved from yeast to human as described in section 2.5, which indicates that it is possible to study several other epigenetic modifications than DNA methylation in *S. cerevisiae*. The genome size of *S. cerevisiae* is also smaller than the genome size of mammalian cells (Dolinski & Botstein, 2007), which for example would make it easier to determine the specific epigenetic alterations responsible for the phenotypic changes in this study. All these properties of *S. cerevisiae* implies that it is efficient to begin to study nutritional epigenetics in *S. cerevisiae* and thereafter move on to higher eukaryotes, which are more similar to humans.

This study showed phenotypic changes of *S. cerevisiae*, which was induced by iron and zinc deficiency and observed as reduced oxidative stress tolerance. These changes were probably caused by epigenetic modifications since the phenotypes with reduced stress resistance were inherited for two or three generations and they were also shown to be reversible. In addition, direct effects of the deficiencies were excluded since the growth rates were restored when the deficient cells were inoculated in non-limited growth medium. These results indicate that *S. cerevisiae* can be used as a model organism to identify nutrients and bioactive food compounds with epigenetic effects, which could be relevant also for humans.

6 CONCLUSION

Previous studies have clearly shown that nutrients can have an impact on the epigenome and hence affect the gene expression and the phenotype of an organism. Since iron and zinc have many essential roles in biological systems, it is most likely that deficiencies of these micronutrients also affects the epigenome. In this study, *S. cerevisiae* with iron deficiency did not show a reduced heat stress tolerance, possibly due to a mutation or an epigenetic modification. However, iron deficiency did induce a phenotype with reduced oxidative stress resistance that was inherited for three generations. The development of zinc deficiency in *S. cerevisiae* resulted in a phenotype with reduced heat stress resistance that was maintained for two generations, but this phenotypic change was not shown to be reversible due to experimental errors. Zinc deficiency also induced a phenotype with reduced oxidative stress resistance that was inherited for two generations.

These results implies that iron deficiency and zinc deficiency can induce inheritable and reversible phenotypes with reduced oxidative stress resistance in *S. cerevisiae*. However, the heat stress experiments need to be repeated before any conclusion can be drawn about epigenetic effects of iron and zinc deficiency related to heat stress resistance. The observed phenotypes with reduced oxidative stress tolerance were most likely caused by epigenetic modifications, but this have to be confirmed in further studies by examining the epigenome on a molecular level. Because of these changes in phenotype, it was also concluded that *S. cerevisiae* can be used as an efficient model organism for studies in nutritional epigenetics, with the goal to improve health and prevent diseases.

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APPENDIX A

The exponential regression lines that were calculated for the exponential growth of *S. cerevisiae* CBS7764 in the different cultures and experiments are shown in Figure 16-21. Measured OD_{600} values from the lag phases were removed and excluded from the regression lines. The specific growth rate of each culture was obtained from the equation of the exponential regression line according to Equation 3.

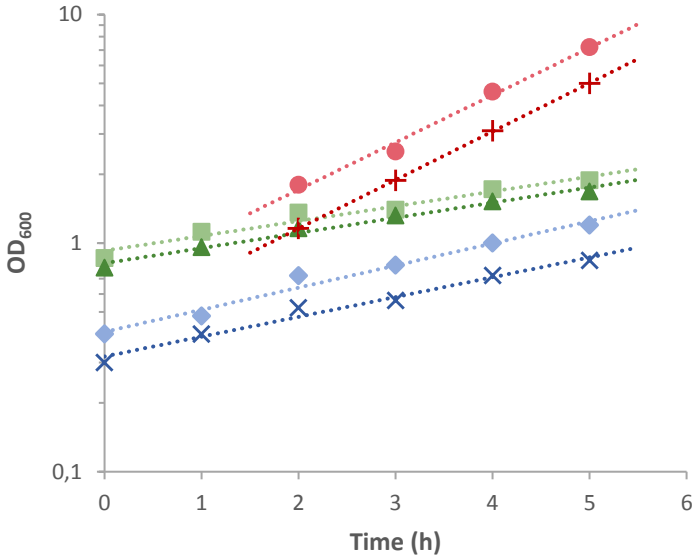

Figure 16. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764 after 2 days in CBS medium containing different concentrations of iron and zinc. (●,✚) control culture 1 and 2 containing 20 µM Zn and 30 µM Fe; (◆) 0.05 µM Fe; (✖) 0.02 µM Fe; (■) 0.1 µM Zn; (▲) 0.03 µM Zn.

Table 7. Equations of the exponential regression lines visualized in Figure 15.

Yeast culture	**Exponential regression line**
Control 1*	$OD_{600} = 0.6615e^{0.4761t}$
Control 2*	$OD_{600} = 0.4365e^{0.4883t}$
0.05 µM Fe	$OD_{600} = 0.4091e^{0.2229t}$
0.02 µM Fe	$OD_{600} = 0.3193e^{0.1996t}$
0.1 µM Zn	$OD_{600} = 0.9262e^{0.1493t}$
0.03 µM Zn	$OD_{600} = 0.8165e^{0.1527t}$

*Containing 20 µM Zn and 30 µM Fe.

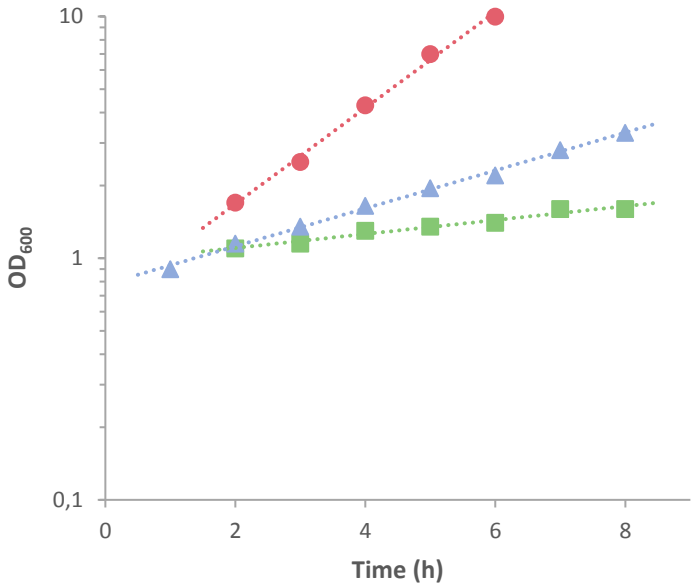

Figure 17. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764 in CBS medium after 32 days of iron and zinc deficiency. (●) control culture containing 20 µM Zn and 30 µM Fe; (▲) 0.01 µM Fe; (■) 0.3 µM Zn.

Table 8. Equations of the exponential regression lines visualized in Figure 16.

Yeast culture	**Exponential regression line**
Control*	$OD_{600} = 0.6713e^{0.4574t}$
0.01 µM Fe	$OD_{600} = 0.7799e^{0.1807t}$
0.3 µM Zn	$OD_{600} = 0.965e^{0.0664t}$

*Containing 20 µM Zn and 30 µM Fe.

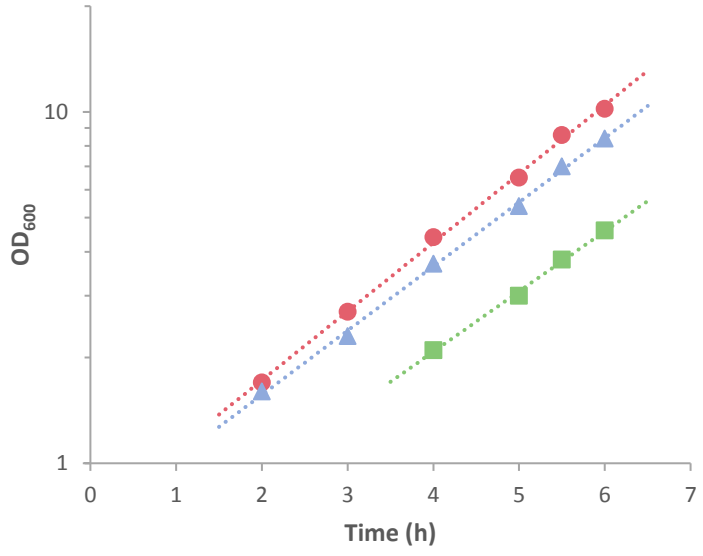

Figure 18. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc, before performing the first epigenetic heat stress experiment. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 9. Equations of the exponential regression lines visualized in Figure 17.

Yeast culture	Exponential regression line
Control*	$OD_{600} = 0.6999e^{0.4507t}$
0.01 µM Fe	$OD_{600} = 0.6752e^{0.4207t}$
0.3 µM Zn	$OD_{600} = 0.428e^{0.3949t}$

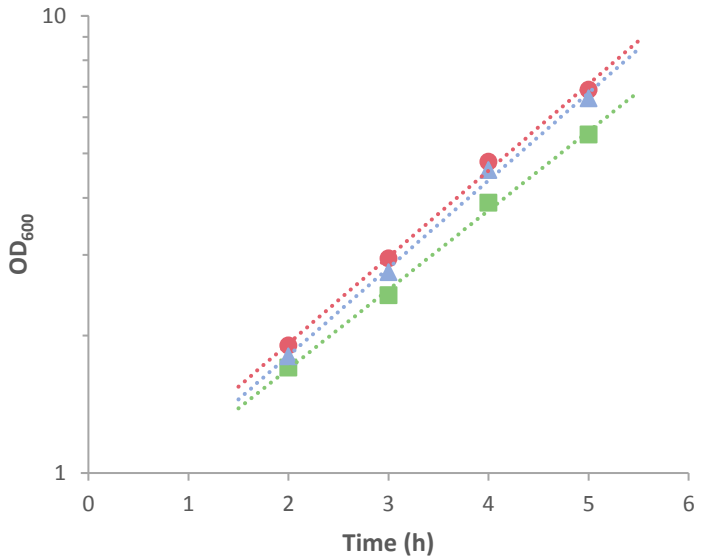

Figure 19. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764, 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc, before performing the second epigenetic heat stress experiment. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 10. Equations of the exponential regression lines visualized in Figure 18.

Yeast culture	Exponential regression line
Control*	$OD_{600} = 0.8037e^{0.4356t}$
0.01 µM Fe	$OD_{600} = 0.7474e^{0.4412t}$
0.3 µM Zn	$OD_{600} = 0.7615e^{0.3987t}$

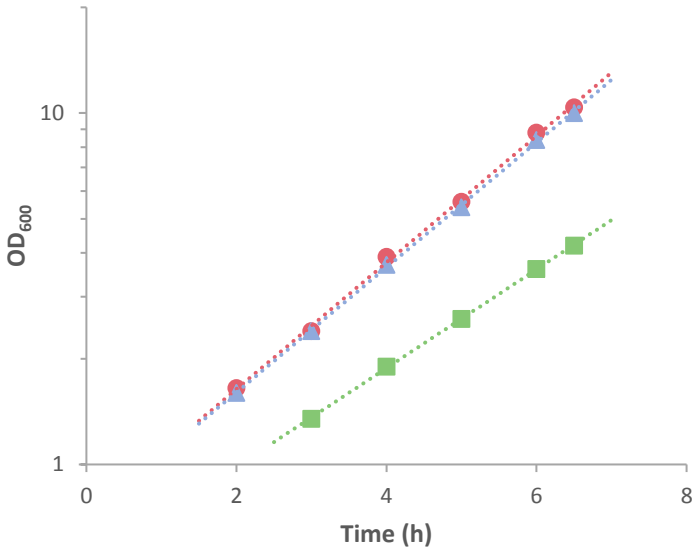

Figure 20. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764 after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc, before performing the first epigenetic oxidative stress experiment. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 11. Equations of the exponential regression lines visualized in Figure 19.

Yeast culture	**Exponential regression line**
Control*	$OD_{600} = 0.7144e^{0.4149t}$
0.01 µM Fe	$OD_{600} = 0.7067e^{0.4096t}$
0.3 µM Zn	$OD_{600} = 0.5155e^{0.3235t}$

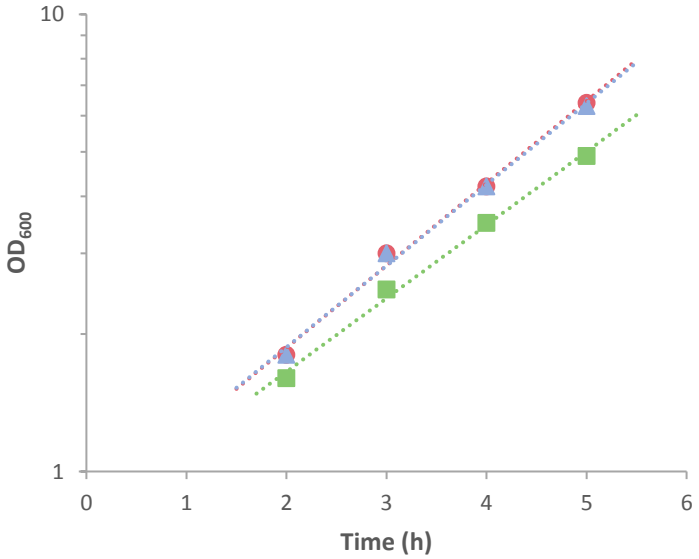

Figure 21. Exponential regression lines calculated for the growth of *S. cerevisiae* CBS 7764, 24 hours after restoring the growth medium to non-limited CBS medium containing 20 µM iron and 30 µM zinc, before performing the second epigenetic oxidative stress experiment. (●) control culture; (▲) culture with previous iron deficiency; (■) culture with previous zinc deficiency.

Table 12. Equations of the exponential regression lines visualized in Figure 20.

Yeast culture	**Exponential regression line**
Control*	$OD_{600} = 0.8144e^{0.4142t}$
0.01 µM Fe	$OD_{600} = 0.8248e^{0.4095t}$
0.3 µM Zn	$OD_{600} = 0.7899e^{0.3694t}$

APPENDIX B

All data from the heat and oxidative stress experiments are presented in Table 13-20, including the number of counted colonies on the YPD plates, the dilution of the spots where the colonies were counted, the mean CFU/ml, the survival after the stress exposures and the standard deviations.

Table 13. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after 10 minutes of heat stress exposure at three different temperatures.

Yeast culture	Temperature (°C)	CFU spot 1	CFU spot 2	CFU spot 3	CFU spot 4	Dilution	Mean CFU/ml	Survival (%)
Control	RT	7	2	4	7	10^4	$5*10^6$	100
Fe	RT	8	4	3	0	10^4	$4*10^6$	100
Zn	RT	8	5	18	8	10^4	$1*10^7$	100
Control	47	7	6	9	5	10^4	$7*10^6$	135
Fe	47	1	2	3	3	10^4	$2*10^6$	60
Zn	47	3	5	1	3	10^4	$3*10^6$	31
Control	50	3	6	7	5	10^4	$5*10^6$	105
Fe	50	1	3	1	4	10^4	$2*10^6$	60
Zn	50	2	0	2	4	10^4	$2*10^6$	21
Control	53	3	2	6	3	10^2	$4*10^4$	1
Fe	53	2	3	2	1	10^2	$2*10^4$	1
Zn	53	2	3	4	1	10^2	$3*10^4$	0

RT, room temperature

Table 14. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. The results are from two independent experiments.

Yeast culture	Time of heat stress (min)	CFU spot 1	CFU spot 2	CFU spot 3	CFU spot 4	Dilution	Mean CFU/ml	Survival (%)	Standard deviation
Control 1	0	43	30	43	46	10^3	$4*10^6$	100	
Control 2	0	47	44	45	24	10^3	$4*10^6$	100	
Control 3	0	35	36	41	39	10^3	$4*10^6$	100	
Control 4	0	38	45	46	45	10^3	$4*10^6$	100	0
Fe 1	0	38	32	24	32	10^3	$3*10^6$	100	
Fe 2	0	30	25	32	25	10^3	$3*10^6$	100	
Fe 3	0	58	38	49	33	10^3	$4*10^6$	100	
Fe 4	0	44	46	47	44	10^3	$5*10^6$	100	0
Zn 1	0	9	12	9	8	10^4	$1*10^7$	100	
Zn 2	0	8	8	10	6	10^4	$8*10^6$	100	
Zn 3	0	5	5	12	6	10^4	$7*10^6$	100	
Zn 4	0	8	4	8	7	10^4	$7*10^6$	100	0
Control 1	5	28	38	33	35	10^3	$3*10^6$	83	
Control 2	5	28	32	33	43	10^3	$3*10^6$	85	
Control 3	5	35	33	25	30	10^3	$3*10^6$	81	
Control 4	5	33	33	28	29	10^3	$3*10^6$	71	6
Fe 1	5	32	21	25	23	10^3	$3*10^6$	80	
Fe 2	5	24	45	27	19	10^3	$3*10^6$	103	

Fe 3	5	32	43	29	26	10^3	$3*10^6$	73	
Fe 4	5	53	40	40	31	10^3	$4*10^6$	91	11
Zn 1	5	46	45	43	45	10^2	$4*10^5$	5	
Zn 2	5	54	43	50	43	10^2	$5*10^5$	6	
Zn 3	5	39	31	29	38	10^2	$3*10^5$	5	
Zn 4	5	43	36	35	40	10^2	$4*10^5$	6	1
Control 1	10	12	8	11	11	10^2	$1*10^5$	3	
Control 2	10	8	13	7	14	10^2	$1*10^5$	3	
Control 3	10	6	2	6	9	10^2	$6*10^4$	2	
Control 4	10	8	8	12	7	10^2	$9*10^4$	2	0
Fe 1	10	2	2	2	5	10^2	$3*10^4$	1	
Fe 2	10	2	3	3	2	10^2	$3*10^4$	1	
Fe 3	10	23	13	17	27	10^2	$2*10^5$	4	
Fe 4	10	23	16	24	21	10^2	$2*10^5$	5	2
Zn 1	10	2	1	1	1	10^2	$1*10^4$	0	
Zn 2	10	7	5	1	3	10^2	$4*10^4$	1	
Zn 3	10	0	1	0	0	10^2	$3*10^3$	0	
Zn 4	10	1	4	1	0	10^2	$2*10^4$	0	0
Control 1	15	1	1	3	2	10^2	$2*10^4$	0	
Control 2	15	1	1	2	4	10^2	$2*10^4$	1	
Control 3	15	1	3	2	1	10^2	$2*10^4$	0	
Control 4	15	0	0	1	0	10^2	$3*10^3$	0	0
Fe 1	15	0	0	0	0	10^2	0	0	
Fe 2	15	0	0	1	1	10^2	$5*10^3$	0	
Fe 3	15	0	0	0	0	10^2	0	0	
Fe 4	15	0	0	0	0	10^2	0	0	0
Zn 1	15	0	2	1	2	10^2	$1*10^4$	0	
Zn 2	15	2	1	1	2	10^2	$2*10^4$	0	
Zn 3	15	0	0	0	0	10^2	0	0	
Zn 4	15	0	0	0	0	10^2	0	0	0

Table 15. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. The cells were cultivated in non-limited CBS medium for 6 hours before they were exposed to heat stress.

Yeast culture	**Time of heat stress (min)**	**CFU spot 1**	**CFU spot 2**	**CFU spot 3**	**CFU spot 4**	**Dilution**	**Mean CFU/ml**	**Survival (%)**	**Standard deviation**
Control 1	0	42	48	58	54	10^3	$5*10^6$	100	
Control 2	0	50	45	38	35	10^3	$4*10^6$	100	0
Fe 1	0	40	29	37	28	10^3	$3*10^6$	100	
Fe 2	0	43	36	33	37	10^3	$4*10^6$	100	0
Zn 1	0	47	59	51	48	10^3	$5*10^6$	100	
Zn 2	0	45	43	56	57	10^3	$5*10^6$	100	0
Control 1	5	51	46	38	41	10^3	$4*10^6$	87	
Control 2	5	44	52	43	42	10^3	$5*10^6$	108	10
Fe 1	5	41	38	32	38	10^3	$4*10^6$	111	

Fe 2	5	36	37	31	30	10^3	$3*10^6$	90	11
Zn 1	5	36	42	26	32	10^3	$3*10^6$	66	
Zn 2	5	39	30	40	36	10^3	$4*10^6$	72	3
Control 1	10	23	27	30	27	10^2	$3*10^5$	5	
Control 2	10	21	29	32	31	10^2	$3*10^5$	7	1
Fe 1	10	1	1	0	2	10^2	$1*10^4$	0	
Fe 2	10	2	1	2	1	10^2	$2*10^4$	0	0
Zn 1	10	1	6	4	3	10^2	$4*10^4$	1	
Zn 2	10	5	2	3	4	10^2	$4*10^4$	1	0
Control 1	15	5	4	6	5	10^2	$5*10^4$	1	
Control 2	15	3	2	2	2	10^2	$2*10^4$	1	0
Fe 1	15	1	0	0	0	10^2	$3*10^3$	0	
Fe 2	15	0	0	0	0	10^2	0	0	0
Zn 1	15	0	0	0	0	10^2	0	0	
Zn 2	15	0	0	0	0	10^2	0	0	0

Table 16. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after heat stress exposure at 51 °C for up to 15 minutes. The cells were cultivated in non-limited CBS medium for 29 hours before they were exposed to heat stress.

Yeast culture	**Time of heat stress (min)**	**CFU spot 1**	**CFU spot 2**	**CFU spot 3**	**CFU spot 4**	**Dilution**	**Mean CFU/ml**	**Survival (%)**	**Standard deviation**
Control 1	0	43	39	37	43	10^3	$4*10^6$	100	
Control 2	0	43	46	34	37	10^3	$4*10^6$	100	0
Fe 1	0	34	52	42	40	10^3	$4*10^6$	100	
Fe 2	0	40	38	36	36	10^3	$4*10^6$	100	0
Zn 1	0	43	51	50	38	10^3	$5*10^6$	100	
Zn 2	0	44	42	48	46	10^3	$5*10^6$	100	0
Control 1	5	22	13	17	19	10^3	$2*10^6$	44	
Control 2	5	22	24	24	16	10^3	$2*10^6$	54	5
Fe 1	5	16	14	8	15	10^3	$1*10^6$	32	
Fe 2	5	14	14	13	18	10^3	$1*10^6$	39	4
Zn 1	5	30	25	26	25	10^3	$3*10^6$	58	
Zn 2	5	25	44	39	36	10^3	$4*10^6$	80	11
Control 1	10	2	1	3	5	10^2	$3*10^4$	1	
Control 2	10	3	2	2	3	10^2	$3*10^4$	1	0
Fe 1	10	1	0	1	2	10^2	$1*10^4$	0	
Fe 2	10	2	2	1	0	10^2	$1*10^4$	0	0
Zn 1	10	0	1	1	1	10^2	$8*10^3$	0	
Zn 2	10	0	3	0	1	10^2	$1*10^4$	0	0
Control 1	15	1	0	0	0	10^2	$3*10^3$	0	
Control 2	15	1	0	0	1	10^2	$5*10^3$	0	0
Fe 1	15	0	0	0	0	10^2	0	0	
Fe 2	15	0	1	0	0	10^2	$3*10^3$	0	0
Zn 1	15	0	0	0	1	10^2	$3*10^3$	0	
Zn 2	15	0	0	1	0	10^2	$3*10^3$	0	0

Table 17. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 after 10 minutes exposure to H_2O_2 at concentrations up to 1.5 M. The cells were cultivated in non-limited CBS medium.

Yeast culture	H_2O_2 concentration (M)	CFU spot 1	CFU spot 2	CFU spot 3	CFU spot 4	Dilution	Mean CFU/ml	Survival (%)
Control	0	46	53	44	44	10^3	$5*10^6$	100
Control	0.1	42	39	48	48	10^3	$4*10^6$	95
Control	0.2	32	40	29	46	10^3	$4*10^6$	79
Control	0.3	38	31	35	28	10^3	$3*10^6$	71
Control	0.4	18	18	18	11	10^3	$2*10^6$	35
Control	0.6	8	7	7	8	10^3	$8*10^5$	16
Control	0.8	5	1	2	2	10^2	$3*10^4$	1
Control	1	1	1	2	3	10^2	$2*10^4$	0
Control	1.5	0	0	0	0	10^2	0	0

Table 18. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes.

Yeast culture	Time of H_2O_2 stress (min)	CFU spot 1	CFU spot 2	CFU spot 3	CFU spot 4	Dilution	Mean CFU/ml	Survival (%)	Standard deviation
Control 1	0	54	47	48	34	10^3	$5*10^6$	100	
Control 2	0	44	41	43	44	10^3	$4*10^6$	100	0
Fe 1	0	18	18	19	15	10^3	$2*10^6$	100	
Fe 2	0	48	47	34	23	10^3	$4*10^6$	100	0
Zn 1	0	8	6	4	5	10^4	$6*10^6$	100	
Zn 2	0	7	6	10	5	10^4	$7*10^6$	100	0
Control 1	10	22	26	25	21	10^3	$2*10^6$	51	
Control 2	10	23	34	20	24	10^3	$3*10^6$	59	4
Fe 1	10	8	10	10	4	10^3	$8*10^5$	46	
Fe 2	10	7	8	4	2	10^3	$5*10^5$	14	16
Zn 1	10	27	31	31	33	10^3	$3*10^6$	53	
Zn 2	10	40	48	37	30	10^3	$4*10^6$	55	1
Control 1	20	19	17	15	20	10^3	$2*10^6$	39	
Control 2	20	23	18	11	16	10^3	$2*10^6$	40	0
Fe 1	20	2	1	2	0	10^3	$1*10^5$	7	
Fe 2	20	0	0	0	1	10^3	$3*10^4$	1	3
Zn 1	20	2	1	2	0	10^3	$1*10^5$	2	
Zn 2	20	8	7	4	6	10^3	$6*10^5$	9	3
Control 1	30	11	9	11	7	10^3	$1*10^6$	21	
Control 2	30	7	5	4	7	10^3	$6*10^5$	13	4
Fe 1	30	0	0	0	0	10^3	0	0	
Fe 2	30	0	0	0	0	10^3	0	0	0
Zn 1	30	0	0	0	2	10^3	$5*10^4$	1	
Zn 2	30	0	1	0	0	10^3	$3*10^4$	0	0
Control 1	60	1	0	0	0	10^3	$3*10^4$	1	

Control 2	60	0	2	2	1	10^3	$1*10^5$	3	1
Fe 1	60	0	0	0	0	10^3	0	0	
Fe 2	60	0	0	0	0	10^3	0	0	0
Zn 1	60	0	0	0	0	10^3	0	0	
Zn 2	60	0	0	0	0	10^3	0	0	0

Table 19. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes. The cells were cultivated in non-limited CBS medium for 6.5 hours before they were exposed to oxidative stress.

Yeast culture	**Time of H_2O_2 stress (min)**	**CFU spot 1**	**CFU spot 2**	**CFU spot 3**	**CFU spot 4**	**Dilution**	**Mean CFU/ml**	**Survival (%)**	**Standard deviation**
Control 1	0	30	44	38	41	10^3	$4*10^6$	100	
Control 2	0	46	49	29	48	10^3	$4*10^6$	100	0
Fe 1	0	33	33	36	46	10^3	$4*10^6$	100	
Fe 2	0	35	47	48	45	10^3	$4*10^6$	100	0
Zn 1	0	44	43	44	53	10^3	$5*10^6$	100	
Zn 2	0	42	48	44	37	10^3	$4*10^6$	100	0
Control 1	10	*	*	*	*	*	*	*	
Control 2	10	27	32	33	27	10^3	$3*10^6$	69	0
Fe 1	10	6	12	7	11	10^3	$9*10^5$	24	
Fe 2	10	9	8	6	6	10^3	$7*10^5$	17	4
Zn 1	10	15	22	28	23	10^3	$2*10^6$	48	
Zn 2	10	8	14	13	10	10^3	$1*10^6$	26	11
Control 1	20	15	22	24	19	10^3	$2*10^6$	52	
Control 2	20	25	12	25	26	10^3	$2*10^6$	51	2
Fe 1	20	5	4	7	5	10^3	$5*10^5$	14	
Fe 2	20	7	6	5	6	10^3	$6*10^5$	14	2
Zn 1	20	15	12	11	9	10^3	$1*10^6$	26	
Zn 2	20	12	18	11	10	10^3	$1*10^6$	30	0
Control 1	30	13	15	10	17	10^3	$1*10^6$	36	
Control 2	30	16	14	12	13	10^3	$1*10^6$	32	1
Fe 1	30	0	0	0	2	10^3	$5*10^4$	1	
Fe 2	30	2	3	2	2	10^3	$2*10^5$	5	0
Zn 1	30	5	3	3	5	10^3	$4*10^5$	9	
Zn 2	30	2	1	3	6	10^3	$3*10^5$	7	2
Control 1	60	0	0	0	1	10^3	$3*10^4$	1	
Control 2	60	1	1	0	0	10^3	$5*10^4$	1	0
Fe 1	60	0	0	0	0	10^3	0	0	
Fe 2	60	0	0	0	0	10^3	0	0	0
Zn 1	60	0	0	1	0	10^3	$3*10^4$	1	
Zn 2	60	1	1	0	0	10^3	$5*10^4$	1	0

*Missing values due to a lot of gas bubbles in the samples which affected the sample volumes.

Table 20. Number of counted colonies and survival of *S. cerevisiae* CBS 7764 with previous iron and zinc deficiency after exposure to 0.5 M H_2O_2 for up to 60 minutes. The cells were cultivated in non-limited CBS medium for 29 hours before they were exposed to oxidative stress.

Yeast culture	Time of H_2O_2 stress (min)	CFU spot 1	CFU spot 2	CFU spot 3	CFU spot 4	Dilution	Mean CFU/ml	Survival (%)	Standard deviation
Control 1	0	45	49	46	44	10^3	$5*10^6$	100	
Control 2	0	42	36	38	50	10^3	$4*10^6$	100	0
Fe 1	0	52	47	57	45	10^3	$5*10^6$	100	
Fe 2	0	44	58	47	53	10^3	$5*10^6$	100	0
Zn 1	0	42	57	53	55	10^3	$5*10^6$	100	
Zn 2	0	51	44	46	46	10^3	$5*10^6$	100	0
Control 1	10	31	32	28	29	10^3	$3*10^6$	65	
Control 2	10	46	27	38	35	10^3	$4*10^6$	88	11
Fe 1	10	37	38	38	42	10^3	$4*10^6$	77	
Fe 2	10	28	37	35	31	10^3	$3*10^6$	65	6
Zn 1	10	30	40	38	40	10^3	$4*10^6$	71	
Zn 2	10	32	32	28	30	10^3	$3*10^6$	65	3
Control 1	20	24	26	19	27	10^3	$2*10^6$	52	
Control 2	20	32	37	26	27	10^3	$3*10^6$	73	11
Fe 1	20	34	28	22	35	10^3	$3*10^6$	59	
Fe 2	20	32	27	25	22	10^3	$3*10^6$	52	3
Zn 1	20	31	36	30	29	10^3	$3*10^6$	61	
Zn 2	20	33	32	44	33	10^3	$4*10^6$	76	8
Control 1	30	23	22	20	19	10^3	$2*10^6$	46	
Control 2	30	17	28	20	29	10^3	$2*10^6$	57	5
Fe 1	30	24	19	21	24	10^3	$2*10^6$	44	
Fe 2	30	22	24	21	16	10^3	$2*10^6$	41	1
Zn 1	30	22	18	19	18	10^3	$2*10^6$	37	
Zn 2	30	20	23	22	23	10^3	$2*10^6$	47	5
Control 1	60	1	2	2	3	10^3	$2*10^5$	4	
Control 2	60	2	2	4	4	10^3	$3*10^5$	7	1
Fe 1	60	2	1	2	5	10^3	$3*10^5$	5	
Fe 2	60	3	0	1	0	10^3	$1*10^5$	2	2
Zn 1	60	2	1	4	4	10^3	$3*10^5$	5	
Zn 2	60	2	2	0	0	10^3	$1*10^5$	2	1